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The effect of ionising radiation on mussels

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**UNIVERSITY OF
PLYMOUTH**

THE EFFECT OF IONISING RADIATION ON MUSSELS

By

EMILY LAURA VERNON

A thesis submitted to the University of Plymouth in
partial fulfilment for the degree of
DOCTOR OF PHILOSOPHY

School of Biological and Marine Sciences

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I dedicate this thesis to my Mum and Dad, you made this possible.

I love you both dearly.

AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award without prior agreement of the Doctoral College Quality Sub-Committee. Work submitted for this research degree at the University of Plymouth has not formed part of any other degree either at the University of Plymouth or at another establishment.

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ABSTRACT

The effect of ionising radiation on mussels.

Emily Laura Vernon

Ionising radiations have undoubtedly played a vital role in modern society. There is however growing concern of their increasing presence in the environment whether by permitted or accidental release, along with other contaminants. While the effects of ionising radiation (IR) on human and mammalian models are well studied, the impacts on aquatic organisms, which play important roles for ecosystem sustainability are yet to be fully understood. This is particularly for chronic, low dose, environmentally relevant exposures, bearing in mind that many of the discharged radionuclides have long half-lives, to which biota are exposed. In this context a multi-biomarker, multi-species approach was adopted to investigate IR-induced (phosphorus-32, ^{32}P) response, alone and in combination with copper (Cu, an environmentally ubiquitous metal), in two ecologically relevant bivalve species, the marine *Mytilus galloprovincialis* (MG) and freshwater *Dreissena polymorpha* (DP) under laboratory conditions. The chosen species play integral roles (ecological, economic and environmental) within coastal and freshwater bodies. Accumulation patterns of Cu (18, 32, 56 $\mu\text{g L}^{-1}$) and ^{32}P (0.10, 1, and 10 mGy d^{-1}) in isolation varied between the species and tissues. In turn, dose rates (^{32}P) to specific tissues were found to exceed those established for the whole-body for these species. This work demonstrated the importance of determining dose rate at tissue level, and highlighted digestive gland and gill as key tissues of interest for subsequent biological assays. In terms of biomarker responses for DNA damage, given that DNA is an important target

for the actions of IR, induction of novel biomarker gamma H2AX (γ -H2AX) was performed alongside more classical techniques, including comet and micronucleus (MN) assays, along with molecular approaches (i.e. transcriptional expression of key genes involved in stress responses) and behavioural level changes. Genotoxicity was well correlated with Cu concentration, and ^{32}P dose rate, both as single and combined stressors. Significant DNA damage was noted at $32\text{ }\mu\text{g L}^{-1}$ (Cu), and 1 mGy d^{-1} (^{32}P). In terms of relative sensitivity, overall, MG appeared more sensitive for the induction of γ -H2AX and DNA strand breaks (comet assay), both biomarkers of exposures. In contrast, DP was found to be more susceptible for the induction of MN (biomarker of effects) in the target tissues. The study also highlights that a single screening dose rate may not be adequate to protect all species. The integrated, multi-biomarker, species and tissue approach adopted in the current study provides a thorough, robust methodology which could be further applied to other ecologically relevant species, or reference organisms. The study contributes to the limited amount of information related to understanding of IR-induced biological responses on aquatic biota, alone and in combination with a relevant metallic contamination. It goes some way towards providing the necessary scientific basis for the development of adequate protective policies for both coastal and inland water bodies.

TABLE OF CONTENTS

ACKNOWLEDGMENTS	I
AUTHORS DECLARATION	III
ABSTRACT	V
LIST OF FIGURES	XIII
LIST OF TABLES	XXIII
ABBREVIATIONS, SYMBOLS AND ACRONYMS	XXVI
 Chapter 1: General introduction	 1
1.1 Environment and human health	3
1.2 Ionising radiation	4
1.2.1 Radioactivity in the aquatic environment	4
1.2.2 Relative Biological Effectiveness (RBE)	5
1.2.3 Radiation types and ecological relevance	6
1.3 Levels of biological organisation	10
1.3.1 Radionuclide bioaccumulation and dosimetry	10
1.3.2 Biological end points	11
1.4 Aquatic organisms as bioindicators	18
1.4.1 Marine and freshwater bivalves: Use in ecotoxicology	21
1.5 Experimental design: Laboratory or field, and external factors	26
1.6 Overall aims of research	28
1.7 Hypothesis	29
 Chapter 2: Methods and method development	 31
2.1 Chemicals and suppliers	32
2.2 Mussel collection and maintenance	32
2.2.1 Genetic identification of <i>Mytilus</i> spp.	36
2.3 Cellular extractions for biological assays	39
2.3.1 Haemolymph extraction	39
2.3.2 Tissue digestion	40
2.4 Cell viability assessment with Trypan blue	41
2.5 Biological assays	42
2.5.1 Micronucleus (MN) assay	42
2.5.2 Comet assay	44

2.5.2.1 Optimisation and validation of the comet assay using hydrogen peroxide (H ₂ O ₂).....	46
2.5.3 53BP1 and γ-H2AX assays	48
2.5.3.1 Validation of the γ-H2AX assay using H ₂ O ₂ as a reference agent	54
2.5.4 Transcriptional expression of key genes	55
2.5.4.1 RNA extraction.....	57
2.5.4.2 cDNA synthesis.....	57
2.5.4.3 Real-time Polymerase chain reaction (qPCR).....	58
2.5.4.4 Data analysis and interpretation.....	58
2.6 Physiological observations.....	59
2.6.2 Byssal attachment and valve activity	59
2.7 Determination of metal concentration using ICP-OES and ICP-MS.....	61
2.7.1 Tissue preparation.....	61
2.7.2 Determination of metal in water samples	61
2.8 Ionising Radiation	62
2.8.1 Selection of radionuclide and determination of radiation dose levels	62
2.8.2 Radiation safety and experimental design.....	64
2.8.3 Liquid scintillation counting (LSC)	65
2.8.3.1 Tissue preparation for LSC.....	65
2.8.3.2 Liquid scintillation counting: Analysis of water and mussel tissue samples.....	68
2.8.4 Dosimetry calculations and the ERICA tool	69
2.8.5 Optimisation experiments	72
2.8.5.1 Pilot study.....	72
2.8.5.2 LSC optimisation study.....	76
2.9 Statistical analysis.....	78

Chapter 3: Assessing relative sensitivity of marine and freshwater bivalves following exposure to copper: Application of classical and novel genotoxicological biomarkers	81
3.1 Introduction	83
3.2 Materials and methods.....	86
3.2.1 Chemicals and suppliers	86
3.2.2 Mussel exposure conditions	87
3.2.3 Sampling procedures	89

3.2.4 Biological assays	90
3.2.4.1 Isolation of gill cells for genotoxicity assays.....	90
3.2.4.2 Comet assay to determine DNA strand breaks.....	90
3.2.4.3 Analysis of micronuclei (MN) formation.....	90
3.2.4.4 Induction of γ -H2AX foci.....	90
3.2.5 Determination of Cu concentration in soft tissues and in water samples	91
3.2.5.1 Copper (Cu) analysis in tissues using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).....	91
3.2.5.2 Determination of Cu in water samples using ICP-MS.....	91
3.2.6 Statistical analysis	91
3.3 Results	92
3.3.1 Tissue specific Cu accumulation	92
3.3.2 Genotoxic effects and repair capacity in mussel gill cells	95
3.4 Discussion.....	98
3.4.1 Tissue specific Cu accumulation	98
3.4.2 Cu induced genotoxicity in gill cells	100
3.5 Conclusions	105
 Chapter 4: Relative comparison of tissue specific bioaccumulation and radiation dose estimation in marine and freshwater bivalves following exposure to phosphorus-32.....	
4.1 Introduction	109
4.2 Materials and methods.....	112
4.2.1 Chemicals and suppliers	112
4.2.2 Mussel exposure conditions	113
4.2.3 Sampling procedures and liquid scintillation counting	114
4.2.4 Dosimetry and the ERICA TOOL.....	114
4.2.5 Statistical analysis	115
4.3 Results	115
4.3.1 Activity concentrations in water	115
4.3.2 Activity concentrations in bivalve soft tissue, shell and IMW.....	116
4.3.2.1 Soft tissue.....	118
4.3.2.2 Internal mussel water (IMW) and shell.....	119
4.3.2.3 Tissue specific ^{32}P accumulation.....	119

4.3.2.4 Faecal matter and pseudofaeces.....	120
4.3.3 Dosimetry	121
4.4 Discussion	123
4.5 Conclusions	129

Chapter 5: Assessing relative biomarker responses in marine and freshwater bivalve molluscs following exposure to phosphorus 32 (³²P):

Application of genotoxicological and molecular biomarkers	131
5.1 Introduction	133
5.2 Materials and methods.....	136
5.2.1 Chemicals and suppliers	136
5.2.2 Mussel exposure conditions	136
5.2.3 Sampling procedures	139
5.2.4 Biological assays.....	140
5.2.4.1 Isolation of digestive gland and gill cells.....	140
5.2.4.2 Comet assay to determine DNA strand breaks.....	140
5.2.4.3 Analysis of micronuclei induction.....	140
5.2.4.4 Induction of γ-H2AX foci.....	140
5.2.4.5 Determination of transcriptional levels of key genes.....	141
5.2.5 Determination of ³² P and Cu concentration in water samples	141
5.2.5.1 Determination of ³² P in water samples using liquid scintillation counting.....	141
5.2.5.2 Determination of Cu concentration in water samples using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).....	141
5.2.4 Dosimetry and the ERICA tool.....	141
5.2.5 Statistical analysis	142
5.3 Results.....	143
5.3.1 Genotoxic response following <i>in vivo</i> exposures to ³² P	143
5.3.1.1 Comet assay to determine DNA strand breaks.....	145
5.3.1.2 Analysis of micronuclei (MN) induction.....	145
5.3.1.3 Induction of γ-H2AX foci.....	145
5.3.2. Transcriptional expression of key genes	146
5.3.3 Whole-body and tissue specific dose rates	150
5.4 Discussion	150
5.4.1 Dosimetry and dose-response relationship	150
5.4.2 ³² P induced genotoxic response in gill and digestive gland cells.....	151

5.4.2.1 Comet assay to determine DNA strand breaks.....	151
5.4.2.2 Induction of γ -H2AX foci.....	153
5.4.2.3 Analysis of micronuclei (MN) induction.....	155
5.4.3 Expression of key genes	156
5.4.4 Environmental implications and future research.....	158
5.5 Conclusions	159

Chapter 6: Evaluation of interactive effects of phosphorus-32 and copper on marine and freshwater bivalve molluscs.....161

6.1 Introduction	163
6.2 Materials and methods.....	167
6.2.1 Chemicals and suppliers	167
6.2.2 Mussel exposure conditions	167
6.2.3 Sampling procedures.....	168
6.2.4 Biological assays	169
6.2.4.1 Isolation of digestive gland and gill cells.....	169
6.2.4.2 Comet assay to determine DNA strand breaks.....	169
6.2.4.3 Analysis of micronuclei (MN) induction.....	169
6.2.4.4 Induction of γ -H2AX foci.....	169
6.2.4.5 Determination of transcriptional expression of key genes....	170
6.2.5 Behavioural observations: Valve movement and byssus attachment.....	170
6.2.6 Water quality measurements and ^{32}P and Cu analyses	170
6.2.6.1 Determination of ^{32}P in water samples using liquid scintillation counting.....	170
6.2.6.2 Determination of Cu concentration in water samples using Inductively Coupled Plasma Mass Spectrometry (ICP-MS).....	171
6.2.7 Dosimetry and the ERICA TOOL.....	171
6.2.8 Statistical analysis	172
6.2.8.1 Multivariate analysis.....	172
6.3 Results	173
6.3.1 Genotoxic response following <i>in vivo</i> exposures to ^{32}P and Cu.....	174
6.3.1.1 Comet assay to determine DNA strand breaks.....	178
6.3.1.2 Analysis of micronuclei (MN) formation.....	178
6.3.1.3 Induction of γ -H2AX foci.....	179
6.3.2 Transcriptional expression of key genes	179

6.3.3 Multivariate analysis of biomarker reactions.....	181
6.3.4 Behavioural observations: Valve movement and byssus attachment.....	182
6.4 Discussion	184
6.4.1 Biomarker interactions.....	184
6.4.2 ³² P induced genotoxic response in gill and digestive gland cells.....	185
6.4.3 Transcriptional expression of key genes	189
6.4.4 Behavioural observations: Valve movement and byssus attachment.....	191
6.4.5 Environmental implications and future research.....	192
6.5 Conclusions	193
Chapter 7: General discussion and future perspectives	195
7.1 Environmental radiation and dosimetry.....	197
7.1.1 Radiation dosimetry.....	197
7.2 Laboratory vs. field exposures	199
7.3 Mussels as bioindicators of environmental health.....	201
7.3.1 <i>M. galloprovincialis</i> and <i>D. polymorpha</i>	201
7.3.2 Life history stage, transgenerational effects and epigenetics	203
7.4 Suitable biomarkers and radiation science	205
7.4.1 Advancements in radiological research: The development of novel biomarkers	206
7.4.1.1 Proteomics.....	206
7.4.1.2 Metabolomics.....	208
7.5 Conclusions	210
APPENDICES	213
University of Plymouth courses	213
External courses.....	213
Presentations and posters.....	213
Press releases.....	215
Radiation protection document I: Bioaccumulation experiment.....	216
Radiation protection document II: Biological end-point experiments	234
List of accepted publications	248
Radiation hazard map.....	249
References	250

LIST OF FIGURES

Figure 1.1.	Penetration and shielding of the different types of IR.....	[7]
Figure 1.2.	Levels of biological organisation.....	[12]
Figure 1.3.	Direct and indirect IR-induced DNA damage.....	[13]
Figure 1.4.	<i>M. galloprovincialis</i> from Trebarwith Strand (Left) and <i>D. polymorpha</i> from Bude Canal, North Cornwall, UK (Right), and comparative external features and anatomy.....	[22]
Figure 2.1.	Map to show location of collection sites for <i>M. galloprovincialis</i> (Trebarwith, Cornwall) and <i>D. polymorpha</i> (Bude, Cornwall) mussels in the UK.....	[33]
Figure 2.2.	Cell viability (%) of <i>D. polymorpha</i> : Optimisation of water types. (River, bottled and artificial) for maintenance in the laboratory ($n = 6$).....	[34]
Figure 2.3.	Genetic identification of the <i>Mytilus</i> spp. (A) Positions of PCR primers in the Glu-5' gene in the <i>M. edulis</i> and <i>M. galloprovincialis</i> and (B) sequences of the primers (modified from (Inoue et al. 1995)).....	[37]
Figure 2.4.	Me 15/16 PCR products visualised on agarose gel (<i>Mytilus</i> sp.) (Image: L. Dallas, 2014) (A) <i>Mytilus edulis</i> and (B) <i>Mytilus galloprovincialis</i>	[38]
Figure 2.5.	Haemolymph extraction from adductor muscle (B, red circles) in: <i>M. galloprovincialis</i> (A) and <i>D. polymorpha</i> (C).....	[39]
Figure 2.6.	Different cell types in <i>M. galloprovincialis</i> (left) and <i>D. polymorpha</i> (right): Haemocytes (A) gill cells (B) digestive	

gland cells (C) mantle cells and (D) posterior adductor muscle cells (E) (100 μm scale)..... [41]

Figure 2.7. Cell viability in *M. galloprovincialis* haemocytes using the Trypan blue exclusion method. Red circles surround viable (clear) and non-viable (blue cells)..... [42]

Figure 2.8. Simplified micronuclei (MN) formation (top). Normal cell (A) cell with micronuclei (B) (bottom). Both cells are *M. galloprovincialis* gill cells stained with ethidium bromide. [43]

Figure 2.9. Scoring comet assay slides: *M. galloprovincialis* gill cells: Varying levels of DNA damage in response to hydrogen peroxide exposure (top), head and tail of comet (bottom)..... [45]

Figure 2.10. DNA damage (% Tail DNA) in *M. galloprovincialis* (A, B and C) and *D. polymorpha* (D, E and F) haemocytes, gill and digestive gland cells, following exposure to varying concentrations of hydrogen peroxide. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data. $n = 4$ [47]

Figure 2.11. Diagram illustrating the recruitment of phosphorylated H2AX following ATM activation (A). Bottom images show: Healthy cell stained with DAPI (B), DAPI stained cell with foci induction (C), and illustration showing cell and green foci (D) (images B and C: *D. polymorpha* gill cells)..... [48]

Figure 2.12. Average number of γ -H2AX foci in *M. galloprovincialis* and *D. polymorpha* gill cells following exposure to varying

	concentrations of hydrogen peroxide for 1 h. SD is standard deviation of mean data. Images show induction of γ -H2AX-foci in <i>D. polymorpha</i> gill cells. $n = 4$	[51]
Figure 2.13.	Optimisation of γ -H2AX assay. Images show microscope image of DAPI stained <i>M. galloprovincialis</i> gill cells, subsequent to varying blocks (Bovine serum albumin [BSA], foetal bovine serum [FBS], milk powder and normal goat serum).....	[53]
Figure 2.14.	Average number of γ -H2AX foci in <i>M. galloprovincialis</i> and <i>D. polymorpha</i> gill cells following exposure to varying concentrations of hydrogen peroxide. SD is standard deviation of mean data. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. $n = 4$	[54]
Figure 2.15.	Diagram illustrating stages of gene expression protocol.	[59]
Figure 2.16.	(A) Byssus thread produced by <i>M. galloprovincialis</i> , (B) <i>M. galloprovincialis</i> filter feeding and (C) <i>D. polymorpha</i> filter feeding.....	[60]
Figure 2.17.	^{32}P shielding: Designs and measurements for shielding (A-C), shielding with beaker covered with acrylic lid in individual compartment (D), safety exposure using fluorescein as surrogate for ^{32}P (E-F).....	[65]
Figure 2.18.	Mechanism behind liquid scintillation counting (LSC).....	[66]
Figure 2.19.	Whole mussel tissue homogenised in different volumes of DI water (5, 10, 20 mL).....	[67]

- Figure 2.20.** Series ^{32}P dilution showing nominal and achieved values. Error bars are SD of mean data..... [69]
- Figure 2.21.** Diagram to show calculation of dose rates in *M. galloprovincialis* and *D. polymorpha*. Ksib and Chi are scaling parameters, representing the lengths of the minor axes in terms of length of the major axis of the ellipsoid. [71]
- Figure 2.22.** ^{32}P accumulation in *M. galloprovincialis* and *D. polymorpha* whole soft tissue, over time. Error bars show standard deviation of mean data. $n = 3$ (MG), 14 (DP). Significance (***) is $p < 0.001$ is shown between species on each sampling day..... [75]
- Figure 2.23.** ^{32}P accumulation in *M. galloprovincialis* and *D. polymorpha* whole soft tissue, shell and internal mussel water. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) between species. Error bars show standard deviation of mean data. $n = 3$ (MG), 14 (DP)..... [77]
- Figure 3.1.** Overall experimental design: Experiment 1 (EXP1): Determination of Genotoxic responses; Experiment 2 (EXP2): Determination of bioaccumulation of copper (Cu)..... [88]
- Figure 3.2.** Tissue specific accumulation of copper in *M. galloprovincialis* (left) and *D. polymorpha* (right), microgram per gram of mussel tissue (dry weight) in control and exposed treatment groups. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001)

from the corresponding control. SD is standard deviation of mean data. $n = 9$ [93]

Figure 3.3. Pearson's correlation analyses of Cu accumulation in gill tissue. Top to bottom: DNA damage (% tail DNA), induction of γ -H2AX foci and micronuclei (MN) formation in *M. galloprovincialis* (left) and *D. polymorpha* (right). $n = 9$.. [94]

Figure 3.4. Genotoxic responses in *M. galloprovincialis* and *D. polymorpha* gill cells following a 10 day exposure to copper (Cu). (A) DNA damage (% tail DNA), (B) Induction of micronuclei (MN) and (C) induction of γ -H2AX foci Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data. Images show (left) control cell and (right) damaged cell. $n = 9$ [96]

Figure 3.5. Pearson's correlation analyses. Top to bottom: % tail DNA and induction of γ -H2AX foci; % tail DNA and induction of MN and induction of MN and γ -H2AX in *M. galloprovincialis* (left) and *D. polymorpha* (right). $n = 9$ [97]

Figure 4.1. Tissue specific accumulation of ^{32}P in *M. galloprovincialis* (MG, left) and *D. polymorpha* (DP, right), total activity per gram of mussel tissue in control and irradiated treatment groups. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. Lower case letters denote variation between similar tissues (species specific, i.e. there is a significant difference in ^{32}P bioconcentration in digestive gland between each

treatment group). Upper case letters denote significant variation in similar tissue and treatment group between species (e.g. MG digestive gland tissue values [0.1, 1 and 10 mGy/d treatments] are significantly different than DP values in the corresponding treatment, there is no difference in control samples). SD is standard deviation of mean data.

IMW – Internal mussel water..... [117]

Figure 4.2. Proportion of ^{32}P in tissue after 10 day exposure in *M. galloprovincialis* (left) and *D. polymorpha* (right). IMW – Internal mussel water..... [118]

Figure 4.3. Activity levels (Bq g^{-1}) in *M. galloprovincialis* and *D. polymorpha* faecal matter (dry weight), following ^{32}P exposure. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. SD is standard deviation of mean data..... [120]

Figure 5.1 Schematic diagram showing experimental design. Images from *M. galloprovincialis* digestive gland cells..... [138]

Figure 5.2 Genotoxic effects in *M. galloprovincialis* and *D. polymorpha* gill cells following a 10-day exposure to ^{32}P . Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. SD is standard deviation of mean data. $n = 9$ [144]

Figure 5.3 Pearson's correlation analyses of: (top to bottom) % tail DNA and induction of MN, % tail DNA and $\gamma\text{-H2AX}$ foci, and induction of MN and $\gamma\text{-H2AX}$ foci. *M. galloprovincialis*

digestive gland (left) and *M. galloprovincialis* gill (right). $n = 9$ [147]

Figure 5.4

Pearson's correlation analyses of: (top to bottom) % tail DNA and induction of MN, % tail DNA and γ -H2AX foci, and induction of MN and γ -H2AX foci. *D. polymorpha* digestive gland (left) and *D. polymorpha* gill (right). $n = 9$ [148]

Figure 5.5

Relative expression ratios (RER) of key genes in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10-day exposure to ^{32}P . Data are normalised for reference genes (*ef1*, *actin*) and controls. Error bars indicate the 95% confidence intervals. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. $n = 9$ [149]

Figure 6.1

Genotoxic effects and subsequent repair in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10 day exposure to ^{32}P and Cu, alone and in combination. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. Letters are indicative of significant differences ($p < 0.05$) between species tissue (i.e. MG gill tissue). SD is standard deviation of mean data. $n = 9$ [175]

Figure 6.2a

Scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data, not including behavioural response. Vectors indicate the directionality of specific biomarkers. (A, B) *M. galloprovincialis* digestive gland and

(C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$)..... [176]

Figure 6.2b

Scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data, not including behavioural response. Vectors indicate the directionality of specific biomarkers. (A, B) *D. polymorpha* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$)..... [177]

Figure 6.3

Relative expression ratios (RER) of key genes in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10-day exposure to ^{32}P and Cu, alone and in combination. Data are normalised for reference genes (ef1, actin) and controls. Error bars indicate the 95% confidence intervals. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. $n = 9$ [180]

Figure 6.4

Behavioural effects in *M. galloprovincialis* (MG) and *D. polymorpha* (DP) following a 10 day exposure to ^{32}P and Cu, alone and in combination. (A) Number of attached mussels per beaker, on days 1, 3, 5, 7, 9 and 11 of exposure, (Ba, b) Average number of mussels (Ba: MG, Bb: DP) actively filtering each experimental day, per beaker. SD is standard deviation of mean data. $n = 3$ [183]

LIST OF TABLES

Table 1.1.	The molecular and genetic effects of IR on aquatic biota: Summary of previous literature. Table includes phyla and species, radiation source (alpha, beta, gamma or other), radiation dose or activity, exposure length, author, publication date and doi..... [8]
Table 1.2.	The molecular and genetic effects of IR on aquatic biota: Summary of previous literature (1971-2018). Table includes phyla and species, endpoints investigated and source of radiation used in exposure (α , alpha; β , beta; γ , gamma; *, other [i.e. Natural]). No. of symbols represents the amount of times each biomarker has been utilised in scientific literature..... [20]
Table 2.1.	Summary of primer pairs used for reference and target genes in <i>M. galloprovincialis</i> and <i>D. polymorpha</i> : Name, abbreviation, primer sequences and accession number. [56]
Table 2.2.	Table outlining properties of phosphorus-32..... [63]
Table 2.3.	Alternative radionuclides considered for experimental design..... [63]
Table 2.4.	Table illustrating custom organism option in the ERICA tool; <i>D. polymorpha</i> digestive gland (DG) and <i>M. galloprovincialis</i> DG, occupancy factors and organism geometry. Ksib and Chi are scaling parameters, representing the lengths of the minor axes in terms of length of the major axis of the ellipsoid..... [70]

Table 2.5.	ERICA tool criteria used for estimating dose rate from water concentrations. K_d is distribution coefficient, CR is concentration ratio..... [72]
Table 2.6.	Nominal dose rates (mGy d^{-1}), total dose (mGy 10 d^{-1}) and required water concentration (Bq L^{-1}) for ^{32}P exposures, using the ERICA tool default criteria..... [73]
Table 2.7.	Weight of <i>M. galloprovincialis</i> and <i>D. polymorpha</i> (g).... [73]
Table 2.8.	Water concentrations (Bq L^{-1}), calculated by the ERICA tool, that give to correct dose rates of 0.1, 1 and 10 mGy d^{-1} in <i>M. galloprovincialis</i> and <i>D. polymorpha</i> [78]
Table 3.1.	Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]) and copper concentrations in water ($\mu\text{g L}^{-1}$). Data are presented as mean \pm standard deviation (SD)..... [89]
Table 4.1.	Activity levels in water samples (Bq L^{-1}) per treatment in <i>M. galloprovincialis</i> and <i>D. polymorpha</i> (SD is standard deviation of mean data). Asterisks (*) denote nominal activity concentrations..... [115]
Table 4.2.	Order of ^{32}P accumulation in soft tissue, shell and IMW in <i>M. galloprovincialis</i> and <i>D. polymorpha</i> individuals, order shows tissue with the highest to lowest bioconcentration (Bq g^{-1}) in all treatment groups..... [116]
Table 4.3.	Table to show (a) the expected dose rates in mGy d^{-1} and $\mu\text{Gy d}^{-1}$ (for the ERICA tool), (b) the water activity concentrations that give the correct dose rate (Bq L^{-1}) for both species *as calculated from preliminary experiments,

(c) the average dose rate achieved in mGy d^{-1} and (d) ERICA tool water activity concentrations that give the correct dose (Bq L^{-1}), corrected to 35 g whole mussel weight/beaker. [121]

Table 4.4. Table to show the expected and achieved dose rates (mGy d^{-1}) in *M. galloprovincialis* and *D. polymorpha* digestive gland using custom geometry in the ERICA tool (Tier 2). [122]

Table 5.1 Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]), copper concentrations in water ($\mu\text{g L}^{-1}$) and ^{32}P concentrations in water (Bq L^{-1}), for both species. Data is presented as mean \pm standard deviation. [139]

Table 5.2 Table to show (a) the expected dose rates in mGy d^{-1} and (b) the average dose rate achieved in *M. galloprovincialis* and *D. polymorpha* whole-body, digestive gland and gill tissue (mGy d^{-1}). [142]

Table 6.1 Table to show (a) the expected dose rates in mGy d^{-1} and (b) the average dose rate achieved in *M. galloprovincialis* and *D. polymorpha* whole-body, digestive gland and gill tissue (mGy d^{-1}). [171]

Table 6.2 Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]), copper concentrations in water ($\mu\text{g L}^{-1}$) and ^{32}P concentrations in water (Bq L^{-1}), for both species. Data is presented as mean \pm standard deviation. [174]

ABBREVIATIONS, SYMBOLS AND ACRONYMS

µg	Microgram
µGy	Microgray
µM	Micromole
γ-H2AX	Gamma Histone 2AX
2D-GE	2 dimensional gel electrophoresis
¹³⁷Cs	Caesium-137
³H	Tritium
³²P	Phosphorus-32
³²S	Sulphur-32
⁹⁰Sr	Strontium-90
Al	Aluminium
ALI	Annual limit on intake
AM	Adductor muscle
ATP	Adenosine triphosphate
BaP	Benzo(a)pyrene
Bq	Becquerel
CA	Chromosomal aberration
CAT	Catalase
Cd	Cadmium
cDna	Complimentary DNA
CF	Concentration factor
CR	Concentration ratio

CPM	Counts per minute
Cu	Copper
DAPI	4',6-diamidino-2-phenylindole, dihydrochloride
DDR	DNA damage response
DG	Digestive gland
DI	Deionised water
DO	Dissolved oxygen
DOC	Dissolved organic carbon
DP	<i>Dreissena polymorpha</i>
DSB	Double strand break
D.W	Dry weight
EA	Environment Agency
ERICA	Environmental Risk from Ionising Contaminants: Assessment and Management
EZ	Exclusion Zone
FASSET	Framework for Assessment of Environmental Impact
FPG	Formamidopyrimidine glycosilase
GBq	Gigabecquerel
GC-MS	Gas chromatography-mass spectrometry
GST	Glutathione-S-Transferase
Gy	Gray
H₂O₂	Hydrogen peroxide
HCl	Hydrochloric acid
Hg	Mercury

HR-MS	High-resolution mass spectrometry
HSP	Heat Shock Protein
IAEA	International Atomic Energy Agency
ICP-MS	Inductively coupled plasma mass spectrometry
ICRP	International Commission on Radiological Protection
IMW	Internal mussel water
IR	Ionising radiation
K	Potassium
LC-MS	Liquid chromatography-mass spectrometry
LD	Lethal dose
LET	Linear energy transfer
LMA	Low melting point agarose
LSC	Liquid Scintillation Counting
MBq	Megabecquerel
ME	<i>Mytilus edulis</i>
MG	<i>Mytilus galloprovincialis</i>
mGy	Milligray
MN	Micronuclei
MoA/s	Mode/s of action
NPP	Nuclear power plant
mRNA	Messenger RNA
MS	Mass spectrometry
mSv	Millisievert
NGS	Next generation sequencing

NMA	Normal melting point agarose
NMR	Nuclear magnetic resonance
OST	Other soft tissue
PAH	Polycyclic aromatic hydrocarbon
PBq	Petabecquerel
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
RAPS	Reference animals and plants
RBE	Relative biological effectiveness
RE	Ravenglass estuary
RER	Relative mRNA expression ratio
RMS	Radioactive materials supervisor
RNASeq	RNA sequencing
ROS	Reactive oxygen species
RPA	Radiation protection advisor
SCE	Sister Chromatid Exchange
SOD	Superoxide dismutase
Sp/p	Species
SSB	Single strand break
Sv	Sieverts
USSR	Union of Soviet Socialist Republics

Chapter 1

General Introduction



1.1 Environment and human health

Environmental quality is inherently and complexly linked with human health and well-being. Anthropogenic environmental pollutants such as radionuclides, metals, biological and physical agents (e.g. thermal stress, hypoxia) can threaten both human and non-human biota through a series of complex transfer exposure pathways and physiological processes (Frumkin 2001; Jha 2004; Jha 2008). For the adequate protection of the environment and its inhabitants it is vital that interactions between environment and organism, at different levels of biological organisation (i.e. molecular to ecosystem levels) are understood. This in turn will both improve tools for decision-making and provide more effective environmental protection policies.

With 71% of the earth covered in water, the aquatic environment and inhabiting organisms undoubtedly play a vast role in ecosystem structure and functioning, offering endless ecological, economic, environmental and recreational services. From an ecological perspective aquatic organisms are an integral part of their environments, and often act as keystone species, providing ecosystem services. As an example, bivalves are known to improve water quality, influence nutrient dynamics and biogeochemical processes and provide habitats and nursery grounds for other aquatic life (Edebo et al. 2000; Borthagaray and Carranza 2007; Petersen et al. 2014). Furthermore, organisms such as fish and shellfish are an important and often vital source of protein for both human and non-human biota, many livelihoods rely on seafood as a means of income. Contaminated food is the main pathway of radionuclides into the human body, environmental contaminants discharged in aquatic ecosystems have the potential to reduce the availability of an invaluable commodity (Howard et al. 2013; Steinhauser et al. 2014).

1.2 Ionising radiation

1.2.1 Radioactivity in the aquatic environment

With applications ranging from nuclear energy generation, diagnostic tools in medical, pharmaceutical, research industries and consumer products, it is clear that radioactive materials contribute significantly to modern day society. Nonetheless radionuclides, in common with other contaminants have the potential to enter the natural environment via various transfer pathways, with aquatic ecosystems being the final recipient for many anthropogenic contaminants (Pentreath 1988; Jha 2004). Although exposed to natural background levels of IR (i.e. cosmic, geological), it is the environmental impact of anthropogenic radionuclides, either by regulated or accidental release that is of a growing concern to society, governments, industry and regulators (Hu et al. 2010).

Certain organisations (e.g. nuclear power industry, research organisations, universities and hospitals) are permitted by authorities to discharge regulated levels of radionuclides into the environment (Hu et al. 2010; IAEA 2010). For example in 2011, 8.92×10^{15} Bq and 2.07×10^{15} Bq of liquid tritium (^3H) was discharged by the nuclear fuel reprocessing plants at La Hague, France and Sellafield, UK, respectively (OSPARcommission 2011). In the same year total liquid discharges of beta-emitters (not including ^3H) from nuclear installations under the OSPAR convention (1992 Convention for the Protection of the Marine Environment of the North-East Atlantic), were 2.59×10^{13} Bq. Approximately 70% was attributed to Sellafield, Springfield's (UK, nuclear fuel production installation) and La Hague (OSPARcommission 2011). Despite public and policy concerns over safety, both human and environmental, nuclear power generation offers a

relatively low cost, low carbon form of energy. Combined depletion of natural resources and rapid population growth will continue to drive the requirement for nuclear production and reprocessing installations.

Radionuclides are also released into the environment via nuclear accidents, such as from Chernobyl, Ukraine (formally USSR, 1986) and Fukushima, Japan (2011), or nuclear weapons testing. Following the nuclear incident at the Fukushima Daiichi nuclear power plant (NPP), resulting from the Tohoku earthquake a wide range of terrestrial and aquatic environments were contaminated (Chino et al. 2011). Contamination occurred from atmospheric dispersion, direct release and discharge with the primary source of highly radioactive water originating from a trench surrounding the NPP (IAEA 2015). Oceanic releases were estimated at 1 – 6 petabecquerel (PBq) for caesium-137 (^{137}Cs) and 10 - 20 PBq for iodine-131 (^{131}I); strontium-90 (^{90}Sr) and caesium-134 (^{134}Cs) were also of concern (Yamaguchi et al. 2014; IAEA 2015).

Considering the influx of radioactive contaminants into the aquatic environment, whether from accidental or permitted release it is vital to quantify potential risks to humans, and potential detrimental biological responses in biota (Dallas et al. 2012). Understanding IR-induced effects on various levels of biological organisation, in a range of biota will help provide the necessary scientific background for radiation protection.

1.2.2 Relative Biological Effectiveness (RBE)

Radiation weighting factors and relative biological effectiveness (RBE) are useful tools in radiobiology. They allow for the evaluation of risks and potential consequences of radioactive contamination on aquatic organisms (Dallas et al.

2012). The extent of IR-induced damage is related to linear energy transfer (LET), this refers to the amount of locally-absorbed energy per unit length (Danzker et al. 1959; Eisler 1994). IR (i.e. alpha, beta and gamma) varies in LET; protons, neutrons, and alpha particles for example have much higher LET than gamma or x-rays (Broerse and Barendsen 1986). The higher the LET, the more damaging is the radiation and the smaller is the dose required to produce a specific biological response. LET is taken under consideration in radiation protection measures, weighting factors and RBE were introduced to account for variation. To determine total effective absorbed dose (in Sieverts, Sv for humans), a total absorbed dose (in Gray, Gy) for a given radiation source is multiplied by a variable factor for specific organs called the RBE. The RBE compares the dose of a test IR to the dose of a reference radiation (typically x-rays or γ -rays), in producing an equal biological response. These biological responses are dependent on variables or confounding factors such as absorbed dose, dose rate, the biological system studied or environmental conditions (EA 2001; Valentin 2003).

1.2.3 Radiation types and ecological relevance

IR is typically grouped by the nature of the particles (particulate radiation) or electromagnetic waves (electromagnetic radiation) that cause the ionisation, consisting of alpha and beta particles, gamma and X- rays (Fig. 1.1), it refers to radiation with enough energy to ionise or remove an electron from an atom. Alpha and beta emissions directly ionise atoms on interaction, gamma rays in comparison create secondary (i.e. beta radiation) electron emissions as it passes through certain materials, which then ionises other atoms. Study radionuclides are often chosen on the basis of ecological relevance, for example prevalence or longevity within the natural environment (Table 1.1).

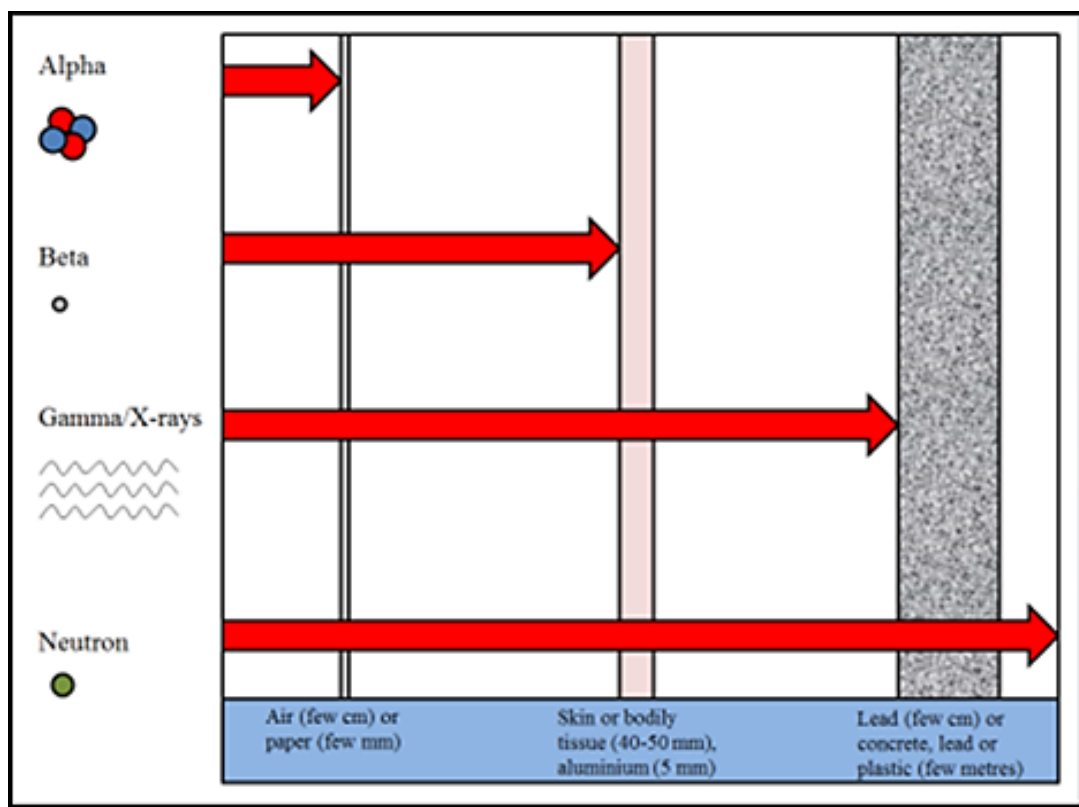


Figure 1.1. Penetration and shielding of the different types of IR.

High-LET alpha decay consists of heavy, short-range particles with two neutrons and two protons being ejected from the nucleus of a radioactive atom. Typically occurring from the heaviest nuclides (Uranium, U; Radium, Ra; Polonium, Po) it is the least penetrating form of radiation, able to travel only a short distance through air. Alpha radiation is most harmful when inhaled (e.g. radon gas), swallowed or absorbed into an organism (Olsvik et al. 2012). Bioaccumulation of alpha emitters, such as Polonium-210 (^{210}Po) has been documented in aquatic organisms including bivalves (Connan et al. 2007; Štok and Smodiš 2011; Feroz Khan et al. 2014), fish (Carvalho et al. 2011; Štok and Smodiš 2011) and cephalopods (Štok and Smodiš 2011), due to the potential threat to human health via consumption. In terms of radiotoxicity research, alpha emitters are underrepresented (Table. 1.1).

Table 1.1. The molecular and genetic effects of IR on aquatic biota: Summary of previous literature. Table includes phyla and species, radiation source (alpha, beta, gamma or other), radiation dose or activity, exposure length, author, publication date and doi.

Phyla/species	Radiation source			Radiation dose/activity	Exposure length	Author, publication date and doi
	Alpha	Beta	Gamma			
Chordates						
<i>Catla Catla</i>			⁶⁰ Co, ¹³⁷ Cs	5 Gy (⁶⁰ Co: 3.2 Gy min ⁻¹ , ¹³⁷ Cs: 0.002 Gy min ⁻¹)	42 h	Anbumani, S and Mohankumar, MN. 2012. doi: 10.1016/j.aquatox.2012.06.001
<i>Catla Catla</i>			⁶⁰ Co, ¹³⁷ Cs	5 Gy (⁶⁰ Co: 3.2 Gy min ⁻¹ , ¹³⁷ Cs: 0.002 Gy min ⁻¹)	42 h	Anbumani, S and Mohankumar, MN. 2015. doi: 10.1016/j.aquatox.2014.11.011
<i>Catla Catla</i>			⁶⁰ Co, ¹³⁷ Cs	5 Gy (⁶⁰ Co: 3.2 Gy min ⁻¹ , ¹³⁷ Cs: 0.002 Gy min ⁻¹)	43 h	Anbumani, S and Mohankumar, MN. 2016. doi: 10.1016/j.aquatox.2016.07.018
<i>Cyprinus carpio</i>			¹³⁷ Cs	0, 2, 4, 6, 8, 10, 20, 30, 40 or 50 Gy	Variable	IK, PK et al. 2015. doi: 10.1016/j.mrgentox.2015.08.005
<i>Danio rerio</i>		³ H		0.4 and 4 mGy h ⁻¹	24 and 96 h	Arcanjo, C et al. 2018. doi: 10.1016/j.aquatox.2018.04.012
<i>Danio rerio</i>			¹³⁷ Co	1, 2, 5, 10 Gy	Variable	Freeman, JL et al. 2014. doi: 10.3389/jene.2014.00268
<i>Danio rerio</i>			¹³⁷ Cs	0.8 and 570 mGy d ⁻¹	96 h	Gagnaire, B et al. 2015. doi: 10.1016/j.aquatox.2015.10.005
<i>Danio rerio</i>			¹³⁷ Cs	0.01, 0.05, 0.1, 0.5 and 1 Gy	N/A	Hu, M et al. 2016. doi: 10.1007/s00411-016-0683-4
<i>Danio rerio</i>			⁶⁰ Co	0.54, 5.4 and 10.9 mGy h ⁻¹	3 h	Hurem, S et al. 2017a. doi: 10.1371/journal.pone.0179259
<i>Danio rerio</i>			⁶⁰ Co	8.7 and 53 mGy h ⁻¹ , 9.6 mGy h ^{-1b}	27 d, 65 h ^a	Hurem, S et al. 2017b. doi: 10.1016/j.envres.2017.07.053
<i>Danio rerio</i>			⁶⁰ Co	8.7 and 53 mGy h ⁻¹	27 d	Hurem, S et al. 2018a. doi: 10.1016/j.envres.2018.02.031
<i>Danio rerio</i>			⁶⁰ Co	8.7 and 53 mGy h ⁻¹	27 d	Hurem, S et al. 2018b. doi: 10.1016/j.envres.2018.02.031
<i>Danio rerio</i>			¹³⁷ Cs	0.1 and 1 Gy	Variable	Jaafar, J et al. 2013. doi: 10.1371/journal.pone.0172023
<i>Danio rerio</i>			¹³⁷ Cs	0.4, 1.2 or 7.2 mGy h ⁻¹	1 or 24 h	Jaafar, J et al. 2013. doi: 10.1371/journal.pone.0172023
<i>Danio rerio</i>			¹³⁷ Cs	100, 500 and 1000 mGy	Variable	Jaafar, J et al. 2013. doi: 10.1371/journal.pone.0172023
<i>Danio rerio</i>			¹³⁷ Cs	1, 10, 100, 750, 1000 mGy d ⁻¹ and 0.3, 1 and 2 Gy d ⁻¹	24 h, acute	Jaafar, J et al. 2013. doi: 10.1371/journal.pone.0172023
<i>Danio rerio</i>			¹³⁷ Cs	0.7, 7, 70, 550 mGy d ⁻¹	24 h	Jaafar, J et al. 2013. doi: 10.1371/journal.pone.0172023
<i>Danio rerio</i>			¹³⁷ Cs	1, 10, 100 and 1000 mGy d ⁻¹	24 h	Jaafar, J et al. 2013. doi: 10.1371/journal.pone.0172023
<i>Danio rerio</i>		²²⁶ Ra		2, 23, 117 Bq L ⁻¹	Variable	Rhee, JS et al. 2012. doi: 10.1002/etc.1873
<i>Gadus morhua</i>				2.5, 5, 7.5, and 10 Gy	Variable	Rhee, JS et al. 2013. doi: 10.1016/j.aquatox.2013.05.006
<i>Kryptolebias marmoratus</i>			¹³⁷ Cs	1, 2, 4, 8, and 10 Gy	20 d	Grygoryev, et al. 2013. doi: 10.1667/RRR3190.1
<i>Kryptolebias marmoratus</i>			¹³⁷ Cs	2.4 and 21 mGy d ⁻¹	Variable	Sayed, AEH et al. 2014. doi: 10.1016/j.mrgentox.2014.01.013
<i>Oryzias latipes</i>			¹³⁷ Cs	15 Gy	Variable	Sayed, AEH et al. 2017. doi: 10.1016/j.mrgentox.2016.11.050
<i>Oryzias latipes</i>			⁶⁰ Co	0.1, 0.5, 2.5, 5 Gy	24 to 1236 s	Tsyuso, O et al. 2011. doi: 10.1016/j.mrgentox.2010.11.001
<i>Oryzias latipes</i>			¹³⁷ Cs	10 Gy	10 Gy/min - varied	Urushihara, Y et al. 2012. doi: 10.1016/j.bbrc.2012.10.128
<i>Oryzias latipes</i>			⁶⁰ Co	1.5, 0.35 and 0.09 mGy h ⁻¹	48 h	Heier et al. L.S. 2013. doi: 10.1016/j.jenvrad.2012.04.004
<i>Salmo salar</i>			⁶⁰ Co	75 mGy	5 h	Olsvik, PA et al. 2009. doi: 10.1016/j.jenvrad.2009.11.004
<i>Salmo salar</i>			⁶⁰ Co	15, 70 and 280 mGy	48 h	Song, Y et al. 2014. doi: 10.1016/j.aquatox.2014.07.021
<i>Salmo salar</i>			⁶⁰ Co	70 mGy	5 h	Song, Y et al. 2016. doi: 10.1016/j.aquatox.2016.03.222
Molluscs						
<i>Corbicula fluminea</i>	U			20, 100, 500 µg L ^{-1a} , 10 µg L ^{-1b}	6, 24, 120 and 240 h ^a , 90 d ^a	Simon, O et al. 2011. doi: 10.1016/j.jenvrad.2011.04.004
<i>Crassostrea gigas</i>			⁶⁰ Co	N/A	N/A	Farcy, E et al. 2007. doi: 10.1016/j.aquatox.2006.11.014
<i>Crassostrea gigas</i>	²⁴¹ Am			6.2 mGy and 20 Gy	6 w and 100 min	Farcy, E et al. 2011. doi: 10.1667/RRR2525.1
<i>Crassostrea gigas</i>		³ H		18 kGy	14 d	Devos, A et al. 2015. doi: 10.1016/j.marpolbul.2015.03.039
<i>Dosinia lupinus</i>			⁶⁰ Co	N/A	18 h	Waker, ST et al. 2000. doi: 10.1016/S0305-0491(00)00266-2
<i>Hydrobia ulida</i>				0.25 to 420 µGy h ⁻¹	Chronic - field	Florou, H et al. 2004. doi: 10.1016/j.jenvrad.2004.01.003
<i>Lymnaea stagnalis</i>				2, 4, 6, 8 and 10 Gy	Chronic - field	Gudkov, D et al. 2016. doi: 10.1016/j.jenvrad.2015.09.004
<i>Meretrix casta</i>			⁶⁰ Co	18 kGy	Variable	Kumar, MKP et al. 2014. doi: 10.1016/j.aquatox.2014.02.007
<i>Mytilus</i> spp.		³ H		0.02 and 21.41 mGy	Varied	Waker, ST et al. 2000. doi: 10.1016/S0305-0491(00)00266-2
<i>Mytilus</i> spp.		³ H		12-485 µGy h ⁻¹	96 h	Hagger, JA et al. 2005. doi: 10.1016/j.aquatox.2005.05.013
<i>Mytilus</i> spp.		³ H		12-485 µGy h ⁻¹	96 h	Jha, AN et al. 2005. doi: 10.1016/j.mrgentox.2005.05.008
<i>Mytilus</i> spp.		³ H		122 and 79 µGy h ⁻¹	7 and 14 d	Jha, AN et al. 2006. doi: 10.1016/j.mrgentox.2006.04.023
<i>Mytilus</i> spp.				0.61 µGy h ⁻¹	Chronic - field	Jaeschke, BC et al. 2011. doi: 10.1016/j.envpol.2010.08.033
<i>Mytilus</i> spp.			¹³⁷ Cs	3.18 ± 0.06 Gy h ⁻¹	70 h	Alamri, OD et al. 2012. doi: 10.1016/j.envpol.2012.04.015
<i>Mytilus</i> spp.		³ H		0.26, 0.45 mGy d ⁻¹	7 d	Jaeschke, BC et al. 2015. doi: 10.1016/j.aquatox.2014.09.007
<i>Paphia malabarica</i>	U		⁶⁰ Co	2, 4, 6, 8 and 10 Gy	Variable	Dallas, LJ et al. 2016. doi: 10.1016/j.jenvrad.2016.07.034
<i>Pastilla tarentina</i>				N/A	Chronic - field	Kumar, MKP et al. 2014. doi: 10.1016/j.aquatox.2014.02.008
<i>Perna perna</i>	²¹⁰ Po			0.02 mGy d ⁻¹	Chronic - field	Florou, H et al. 2004. doi: 10.1016/j.jenvrad.2004.01.003
Arthropod						
<i>Chironomus riparius</i>		³ H		250 or 500 µCi/mL	20 d	Blaylock, BG. 1971. doi: 10.4039/Ent103448-3
<i>Chironomus riparius</i>		³ H		Unknown	Unknown	Blaylock, BG. 1973. Radionuclides in ecosystems.
<i>Daphnia magna</i>			¹³⁷ Cs	0.007 to 35.4 mGy h ⁻¹	75 d	Pariset, F et al. 2015. doi: 10.1016/j.aquatox.2015.03.002
<i>Daphnia magna</i>			⁶⁰ Co	0.41, 1.1, 4.3, 10.7, 42.9 and 106 mGy h ⁻¹	24 and 48 h	Gomes, T et al. 2018. doi: 10.1016/j.aquatox.2018.02.039
<i>Gammarus</i> sp.				N/A	Chronic - field	Florou, H et al. 2004. doi: 10.1016/j.jenvrad.2004.01.003
<i>Gammarus</i> sp.				0.5-5 Gy	Varied	Tsytisugina, VG. 1998. doi: 10.1093/oxfordjournals.rpd.a032223
<i>Idotea ballica</i>				0.5-5 Gy	Varied	Tsytisugina, VG. 1998. doi: 10.1093/oxfordjournals.rpd.a032223
<i>Melita palmaria</i>				N/A	Chronic - field	Florou, H et al. 2004. doi: 10.1016/j.jenvrad.2004.01.003
<i>Paracyclops nanus</i>			¹³⁷ Cs	0, 10, 20, 30, 50, 100 Gy, 400 Gy	2Gy/min	Wong, EJ et al. 2014. doi: 10.1016/j.aquatox.2014.02.010
<i>Tigriopus japonicus</i>			¹³⁷ Cs	0 - 800 Gy, 50, 100, 150, and 200 Gy	2Gy/min	Han, J et al. 2014. doi: 10.1016/j.aquatox.2014.04.005
Annelid						
<i>Glycera clavata</i>				N/A	Chronic - field	Florou, H et al. 2004. doi: 10.1016/j.jenvrad.2004.01.003
<i>Neanthes arenaceodentata</i>			⁶⁰ Co	0.5 to 309 R, 0.04-13 Rh	12 - 24 h	Harrison, FL et al. 1981.
<i>Neanthes arenaceodentata</i>			¹³⁷ Cs	2, 4, 8, and 16 Gy	Up to 3 min	Anderson, SL et al. 1990.
<i>Polychaeta Lyca stopsis</i> sp.				N/A	Chronic - field	Florou, H et al. 2004. doi: 10.1016/j.jenvrad.2004.01.003
Echinoderm						
<i>Paracentrotus lividus</i>			¹³⁷ Cs	0, 50, 100, 150, and 200 Gy	Varied	Han, J et al. 2014. doi: 10.1016/j.aquatox.2014.06.009
Rotifer						
<i>Brachionus koreanus</i>				0.1-5 Gy	10 - 250 s	Matranga V et al. 2010. doi: 10.1007/s10646-009-0444-9

Beta decay, such as that emitted from ^{90}Sr , ^{32}P and ^3H consist of high-energy, high-speed electrons or positrons produced during the conversion of a neutron to proton in the nucleus. In respect to IR-induced molecular and genetic responses in aquatic biota, ^3H is the lone beta-emitter utilised in literature to date (Table 1.1, excluding one study) (Blaylock 1971; Hagger et al. 2005; Jha et al. 2005; Jha et al. 2006; Jaeschke et al. 2011; Devos et al. 2015; Dallas et al. 2016a; Gagnaire et al. 2017; Arcanjo et al. 2018; Pearson et al. 2018). Beta emitter strontium-90 (^{90}Sr , half-life = 28.8 y), arguably one of the most relevant radionuclide contaminants in the aquatic environment, released in significant quantities via fallout from the nuclear industry (i.e. energy generation, nuclear weapons and nuclear incidents) is predominantly unrepresented in aquatic radiobiological research. Due to its ease of mobility, persistence and longevity within the environment it is considered a clear radiological risk (Konovalenko et al. 2016), strontium concentrations have been measured in seaweeds (*Sargassum* spp, *Galaxaura marginata*, Freitas et al. 1988) and fish (*Salmo trutta*, *Esox Lucius*, *Perca fluviatilis*, *Coregonus albula*, Preston et al. 1967; Outola et al. 2009), with factors such as water quality influencing accumulation (Outola et al. 2009), the consequent biological effects in aquatic organisms, however, are currently unknown.

The most penetrating form of radiation, gamma decay refers to a nucleus changing from a higher to lower energy state via the emission of electromagnetic radiation (photons). Gamma radiation is beneficial in allowing an accurate estimation of external dose rate to aquatic organisms under well-defined conditions. Gamma-emitters, namely cobalt-60 (^{60}Co) and ^{137}Cs have been utilised to determine radiosensitivity in a range of aquatic organisms (Table 1.1). This includes bivalves (*C. gigas*, *M. edulis* & *Dosinia lupinus*), polychaetes

(*Neanthes arenaceodentata*), crustaceans (*Daphnia magna*) and fish (*D. rerio*, *S. salar*, *Catla catla*, *Cyprinus carpio*, *Kryptolebias marmoratus*, *Oryzias latipes*) (Harrison 1981, 87; Walker et al. 2000; Olsvik et al. 2010; Farcy et al. 2011; Rhee et al. 2012, 13; Freeman et al. 2014; Song et al. 2014, 16; Kumar et al. 2015, 2017; Parisot et al. 2015; Anbumani and Mohankumar 2016; Hurem et al. 2017, 18). Such radionuclides allow researchers to elucidate potential mechanisms involved in IR-induced biological responses under controlled, simplified experimental settings. To note, radionuclides such as ^{32}P and x-rays can be adopted as a baseline to measure damage from differing radionuclides, or used as a substitute for highly impacting radioactive emissions. Laboratory derived data illustrating chronic IR-induced biological effects, with IR source and dose rate representative of realistic environmental conditions are undoubtedly crucial in understanding and predicting the impact of current and future radiation exposures in the aquatic environment (Pereira et al. 2011; Gudkov et al. 2012; Olsvik et al. 2012; Parisot et al. 2015; Dallas et al. 2016a).

1.3 Levels of biological organisation

1.3.1 Radionuclide bioaccumulation and dosimetry

In radiological studies accurate dosimetry is crucial (Stark et al. 2017). Aquatic organisms can be exposed externally or internally to radionuclides of varying physicochemical forms through water, sediment or via ingestion. The behaviour and fate of radionuclides when accumulated into organs/tissue can differ, resulting in variability of delivered dose. For ease of dose calculation, available assessment tools (i.e. ERICA) often make certain adjustments when determining dose such as (a) radionuclide within the organism is uniformly distributed, therefore calculated dose is for the whole body and (b) organisms are ellipsoidal

in shape. More accurate environmental dosimetry and subsequent protective policies can be achieved in part by determining tissue specific radionuclide uptake and dose, before investigating biological response. It is essential to establish the relationship between exposure, tissue specific uptake, dose rate and effect on the aquatic biota, as varying tissue sensitivity could result in a detrimental biological response at levels presumed to be acceptable. Many approaches have been developed to determine suitable benchmarks or screening dose rates which aim to filter out situations of no concern. As outlined in a thorough review (Andersson et al. 2009), a generic (all species) “no effect” dose rate of $10 \mu\text{Gy h}^{-1}$ (0.24 mGy d^{-1}) has been adopted as a screening value (i.e. ERICA tool), dose rates under this value are thought to result in minimal risk to the individual or population.

1.3.2 Biological end points

IR may cause adverse effects at all levels of biological organisation (Fig. 1.2), from molecular to ecosystem levels (Clements 2000; Dallas et al. 2012). In non-human biota, environmental protection is often deemed successful where no observable effects are seen on ecosystems at a population level, or higher levels of organisation. A conservative approach is to identify relationships between contaminant and organism at an individual level (i.e. molecular, genetic to reproductive level change), such information allows for the development of acceptable levels of natural and anthropogenic radionuclides. Combined data from omics technologies and classical methods (i.e. histopathology, population genetics and ecology) can be linked through bioinformatics to illustrate potential impacts of contaminant exposure in a given organism, or population (Miracle and Ankley 2005; Dallas et al. 2012), such information is extremely valuable.

Developing relationships between biological organisation levels could greatly improve our understanding of IR-induced damage in aquatic organisms, and in turn will allow the development of adequate protection strategies.

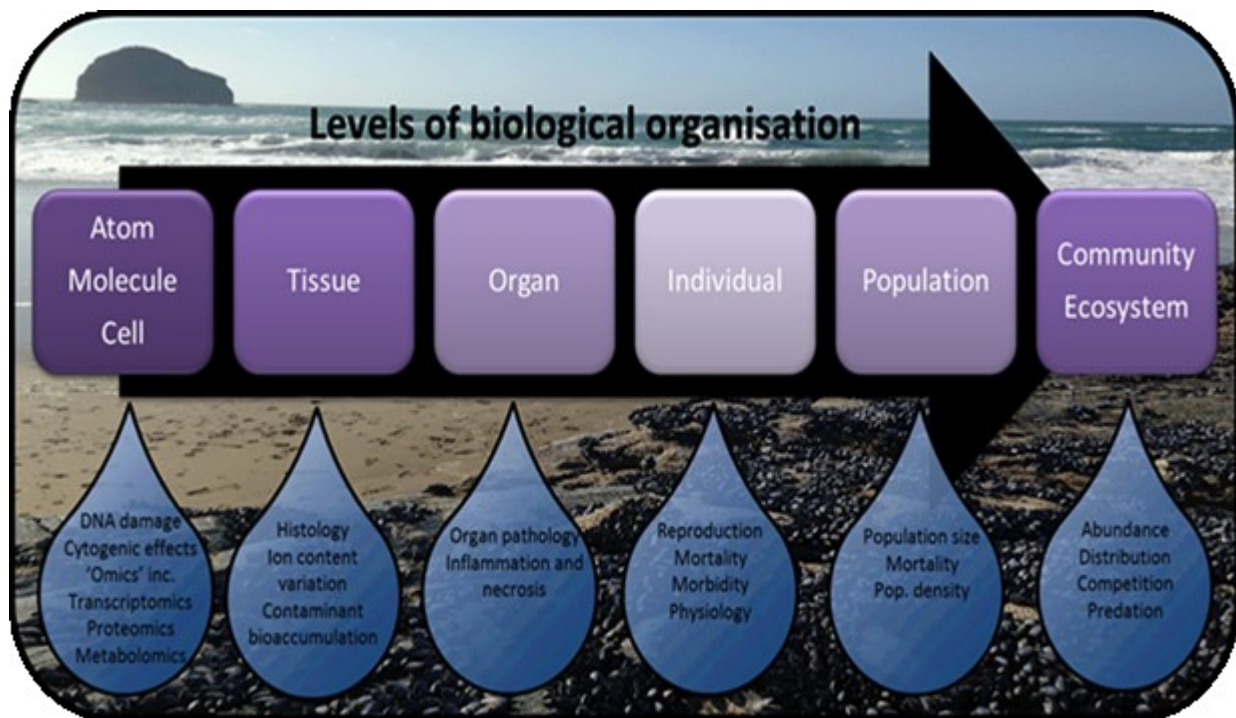


Figure 1.2. Levels of biological organisation.

IR primarily interacts with atoms, while subsequent biological responses may become observable at higher levels of organisation, molecular and genetic variations are often perceived as the first indicators of organism stress. IR is known to cause significant damage to biomolecules; damage can be direct or indirect (Fig. 1.3). Direct toxicity refers to the interaction of radiation with atoms within DNA molecules or to other cellular structures. DNA is considered to be the most important target for the actions of IR (UNSCEAR 1982), and through direct interaction, IR can induce a number of DNA lesions, such as DNA single (SSB) and double strand breaks (DSBs), base lesions and clustered damage (Yokoya et al. 2009) (Fig. 1.3). Indirect effects occur through the generation of reactive

oxygen species (ROS) produced by radiolysis of water. ROS, such as superoxides, hydroxyl and hydrogen peroxide can cause damage to lipids, proteins and DNA, ultimately having an impact on cellular integrity and survival.

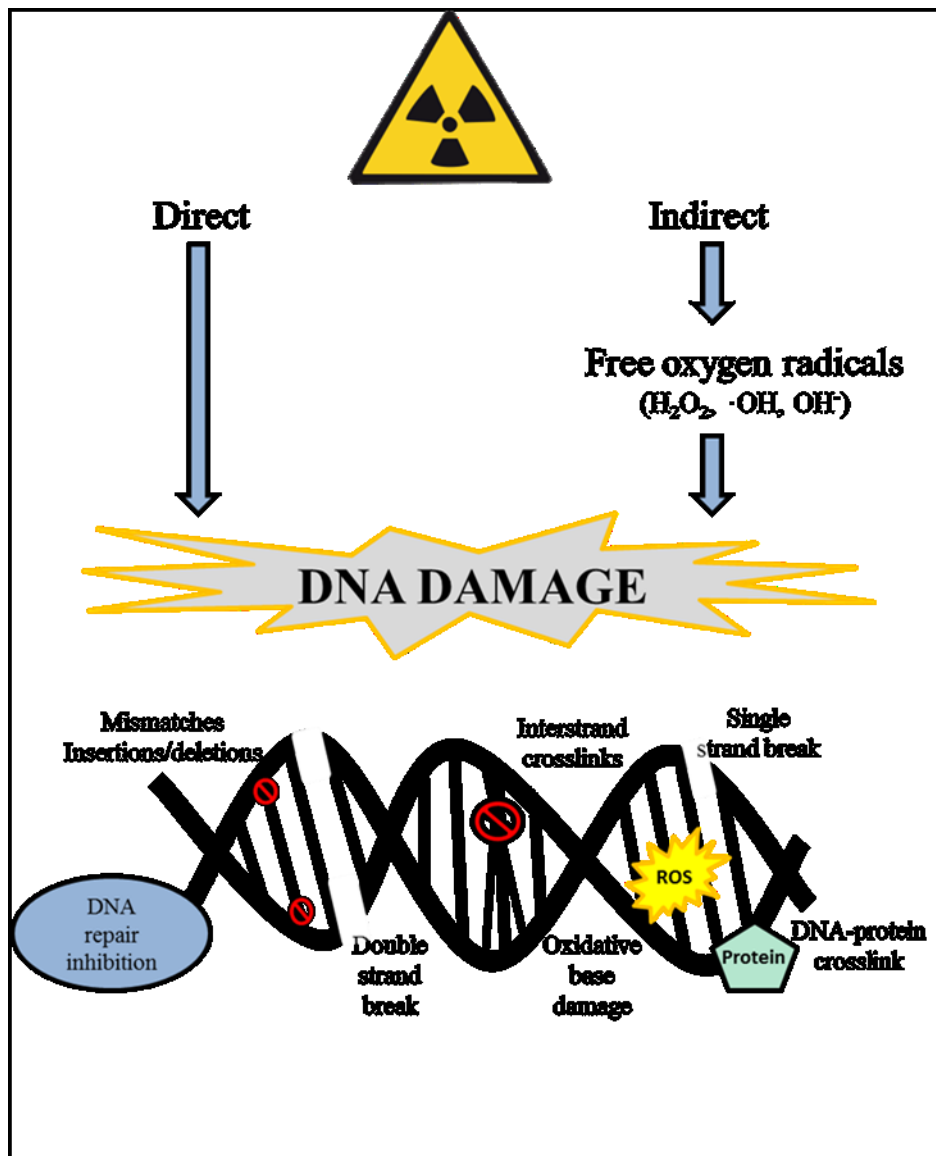


Figure 1.3. Direct and indirect IR-induced DNA damage.

In addition to direct and indirect effects, biological damage can also occur in cells that have not been directly exposed to IR due to proximity to irradiated cells, a

principle coined the bystander effect (Seymour and Mothersill 2004; Mothersill et al. 2006; Mothersill and Seymour 2012; Chevalier et al. 2015). While the nature of the communication system involved in producing a bystander response is not yet fully understood, information from irradiated to neighbouring cells are thought to be transmitted via chemical signalling processes (Seymour and Mothersill 2004). Bystander effects encompass a broad range of damage-mediated endpoints, such as DNA damage, MN, sister chromatid exchange (SCE), chromosomal aberrations (CAs), apoptosis and alterations in gene/protein expression levels (Koturbash et al. 2008; Ilnytsky and Kovalchuk 2011; Choi et al. 2012; Hurem et al. 2017; Burdak-Rothkamm and Rothkamm 2018; Smith et al. 2018a; Smith et al. 2018b). Bystander response has important implications for radiation protection, where effects may also contribute to the final biological consequences of radiation exposure.

Cells have developed numerous defence mechanisms to combat the harmful effects of oxidative damage, such as enzymatic antioxidants (superoxide dismutase [*sod*], catalase [*cat*], glutathione and peroxidases). Such enzymes interact with ROS to convert them into more stable, removable molecules. *Sod*, for example catalyses the breakdown of the superoxide anion into oxygen and hydrogen peroxide. Generation of ROS, along with up/downregulation of antioxidant defences can act as early warning signs, and biomarkers for contaminant-induced oxidative damage. It is important, therefore, to elucidate mechanisms underlying both the production and removal of free radicals in organisms exposed to environmental stressors. Biological assays, such as TBARS (lipid peroxidation) and the enzyme modified comet assay can be utilised to determine oxidative damage in aquatic organisms. The latter, for example, detects DNA bases with oxidative damage with the addition of lesion specific

repair enzymes, such as formamidopyrimidine glycosylase (FPG), endonuclease III and human 8-hydroxyguanine DNA-glycosylase (hOGG1). García-Medina et al (2011), Guilherme et al (2012), Michel and Vincent-Hubert (2012), Dallas et al (2013) and Dallas et al (2016a) successfully utilised this technique in adult bivalves (*M. galloprovincialis*, *D. polymorpha*) and fish (*Anguilla anguilla*, *Cyprinus carpio*), to determine oxidative DNA damage following exposure to IR, metals, aromatic hydrocarbons and organophosphate herbicides.

Cells have a complex range of responses allowing the ability to cope with radiation-induced damage, which rely on molecular level change. Emerging technologies in the field of omics have significant implications for both human health and radiobiological research (Miracle and Ankley 2005). Omics technologies generally refer to (a) transcriptomics, study of the complete set of RNA transcripts produced by the genome, (b) proteomics, measurement of protein levels and most recently (c) metabolomics, the study of endogenous and exogenous low molecular mass metabolites present within a biological system. Gene expression is arguably the first step towards response to any contaminant. Genes, such as *rad51* and *p53* are highly conserved between species, animal models can act as valuable additions to human data. Aquatic species allow for specific examination of certain biological responses, which could not be examined in more complex organisms. Omics tools (e.g. transcriptomics, ecotoxicogenomics), incorporating techniques such as RNA-sequencing (RNASeq) and genome-wide DNA microarrays are increasingly applied in radiation research, and have been widely employed to study the effects of radiation on humans and other mammalian species (i.e. mice, rats), however they have not yet been fully utilised in aquatic organisms (Ogawa et al. 2007; Jaafar et al. 2013; Li et al. 2018). Transcriptomic techniques (i.e. RNASeq, microarrays),

which allow the measurement of expression levels in thousands of genes simultaneously can be utilised to identify early radiation responses, and to aid the development of biomarkers to identify organisms susceptible to radiation, from humans to aquatic organisms.

As levels of mRNA are not directly proportional to the expression level of the proteins they code for, proteomics techniques are arguably more accurately representative of the functional molecules within a cell. The study of proteomics refers to the functional responses of gene expression; the proteins and peptides, along with protein-protein interactions (Connon et al. 2012). It allows a systems-based perspective of how proteins fluctuate, and therefore how aquatic organisms may respond and adapt to various conditions (e.g. natural or anthropogenic) that characterize the aquatic environment (Tomanek 2014). In terms of radiobiological research, the potential advantage of proteomics is not yet fully elucidated. As highlighted by Leszczynski (2014), few studies have examined the proteome in human cells exposed to IR, but due to significant variations in dose rates, exposure conditions and proteomics methods the studies are not comparable.

Regardless of experimental approach, a major drawback in the 'omics' fields is the lack of available annotated genomes, proteomes and metabolomes for most aquatic organisms (Slattery et al. 2012). There is a requirement for large-scale nucleotide sequencing of expressed sequence tags and genomic DNA for organisms chosen for radiation studies. Correlations between 'omics' technologies and more established, validated biomarkers (e.g. DNA or chromosomal damage) should strengthen the certainty of a correct radiation exposure diagnosis in both human and non-human biota.

Whole organism (individual) level effects refer to variations in mortality, physiology, behaviour and reproduction. The Framework for Assessment of Environmental Impact (FASSET) proposed that following IR exposure, morbidity, mortality and reproductive success of the organism should be assessed, such responses were termed 'umbrella endpoints' (Brechignac and Howard 2001). However, once an effect is manifested at an organism level, remedial measures are often too late. While lower levels of biological organisation offer an early warning system, it is difficult to predict the consequential health status of the organism, which will vary depending on factors such as age, sexual maturity and current health (Suter et al. 2005).

Biological responses at population, community or ecosystem levels typically focus on species abundance and diversity, mortality and/or morbidity, species-species interaction (i.e. predation, competition) or alterations in fecundity (Fig. 1.2). Identifying change at higher hierarchical levels offers numerous fundamental advantages over those at lower levels (i.e. molecular, genetic). Such variations are arguably more ecologically relevant due to the incorporation of multiple species, giving an overview of the range of sensitivities to a given contaminant (Attrill and Depledge 1997). However, as with individual effects, once an effect is evident at population or community levels, it is often too late to offer counteractive measures. Furthermore, the complex nature of ecosystem dynamics and function, and influence of biotic and abiotic variation makes the direct route of toxicity to an organism near impossible to elucidate.

In terms of environmental radiation protection, the main level of concern may be populations, communities and ecosystems, however the effect of contaminants are manifested at all levels of biological organisation. With regards to

radiobiological and toxicological studies, there is no 'correct' level at which to study stress, a multi-biomarker approach over several levels of biological organisation will provide insight into the effect of contaminants, its mechanistic base and possible ecosystem wide consequences.

1.4 Aquatic organisms as bioindicators

IR is not an isolated threat, environmental contaminants such as pesticides, metals and natural and synthetic chemicals (Oertel and Salánki 2003) all contribute to the degradation of the aquatic environment. There is a need to assess, monitor and maintain the health status of the natural environment for the benefit of human and non-human biota. Biological monitoring refers to the use of organisms (i.e. plants, animals or microorganisms) or their biological responses (from molecular to individual levels) to determine the current condition or alterations of the environment. Organisms used for biomonitoring are referred to as bioindicator species, biological change within a model system can be used to reflect changes in the natural environment, the presence of contaminants, or to monitor alterations in pollutant levels over time.

Bioindicators share several characteristics. The organism must have good indicator ability, such as the provision of a measureable response proportional to the degree of contamination or degradation (Holt and Miller 2010). It is advantageous for a species to be abundant within an ecosystem for ease of sampling and to aid comparison between locations (Holt and Miller 2010). Finally, it is beneficial for a bioindicator species to be well understood in terms of ecology, life history and to be of economic or commercial relevance (Holt and Miller 2010). In terms of molecular and genetic research, organisms with a complete,

published genome sequences (e.g. *D. rerio*, *D. magna*, *C. gigas*) are advantageous.

Considering the 8.7 million known (2.2 million marine approx., Mora et al. 2013) species on earth, investigating IR-induced biological response in each species is not possible. A summary of phyla and species using in radiation research, with specific focus on IR-induced genetic and molecular effects is displayed on table 1.2, along with specific end-points and radiation source utilised (i.e. alpha, beta, gamma, other). In 2008, the International Commission on Radiological Protection's (ICRP) Committee suggested 12 reference animals and plants (RAPs), defined as “entities that provide a basis for the estimation of radiation dose rate to a range of organisms which are typical, or representative of a contaminated environment” (ICRP 2008). The use of RAPS is beneficial in reducing the current fragmentary nature of radiobiological research by providing focus and uniformity, it aims to reduce the current uncertainty surrounding the biological effects from chronic, low-level exposures to radiation. Databases, such as FREDERICA (Environmental Risk from Ionising Contaminants: Assessment and Management) have been developed in order to collaborate available information and improve understanding of environmental impact of radiations, and to subsequently derive benchmarks of acceptable dose rates considered protective of the structure and function of ecosystems (Copplestone et al. 2008). Bioindicator species for radiation studies include amphibians, aquatic invertebrates, aquatic plants, bacteria, birds, fish, fungi, insects, mammals, mosses/lichens, reptiles, soil fauna, terrestrial plants and zooplankton (Copplestone et al. 2008), representative of freshwater, marine and terrestrial ecosystems.

Table 1.2. The molecular and genetic effects of IR on aquatic biota: Summary of previous literature (1971-2018). Table includes phyla and species, end-points investigated and source of radiation used in exposure (α , alpha; β , beta; γ , gamma; *, other [i.e. Natural]). No. of symbols represents the amount of times each biomarker has been utilised in scientific literature.

Phyla/species	Genetic biomarkers				Biomarkers of oxidative stress				Immunotox. Molecular biomarkers (Gene expression)												
	DNA damage (FbwCyt, DNA-PK)	Apoptosis	Comet assay	Gamma-H2AX	RAPD	MN assay	SCE	Methylation	CA	Lipid peroxidation	Glutathione	SOD	CAT	AChE	Protein-carbonyl content	ROS (e.g. H ₂ DCFDA)	Phenoloxidase activity	RT-PCR, qPCR	Microarray	RNASeq	Proteomics
Chordata																					
<i>Calla calla</i>	V					V												V			
<i>Cyprinus carpio</i>			V			V		V		V	V	V	V	V		VV	V		VV		V
<i>Danio rerio</i>		V	VVVVVV	VV		VV															
<i>Gadus morhua</i>																					
<i>Kryptolebias marmoratus</i>											VV	W									
<i>Oryzias latipes</i>	V	V	V	VV		VV					V								VV	VV	
<i>Salmo salar</i>																			VV	VV	
Molluscs																					
<i>Corbicula fluminea</i>			α																		
<i>Crassostrea gigas</i>			β																$\alpha\beta\gamma\gamma^*$		
<i>Dosinia lupinus</i>															V						
<i>Hydrobia acuta</i>																					
<i>Lymnaea stagnalis</i>																					
<i>Meretrix casta</i>																					
<i>Mytilus</i> spp.			$\alpha\beta\gamma\beta\gamma^*$		β	$\alpha\beta\gamma\beta\gamma$	β								V				β^*		
<i>Paphia malabarica</i>																					
<i>Patella tarentina</i>			V																		
<i>Perna perna</i>																					
Arthropod																					
<i>Chironomus riparius</i>									$\beta\beta$												
<i>Daphnia magna</i>					V					V						V				V	
<i>Gammaridae</i> Gammarus sp.									**												
<i>Idotea baltica</i>									*												
<i>Melita palmata</i>									*												
<i>Paracyclops nana</i>											V	V				V					
<i>Tigriopus japonicus</i>											V					V					
Annelid																					
<i>Grubea clavata</i>									γ^*												
<i>Neanthes arenaceodentata</i>								V													
<i>Polychaeta</i> Lycastopsis sp.									*												
Echinoderm																					
<i>Paracentrotus lividus</i>																					*
Rotifer																					
<i>Brachionus koreanus</i>											V					V				V	

1.4.1 Marine and freshwater bivalves: Use in ecotoxicology

This thesis focused on two mussel species, the marine species *Mytilus galloprovincialis* (MG) and freshwater species *Dreissena polymorpha* (DP). The use of two (or more) species should be considered as a more robust, realistic approach for ecotoxicological studies (Chapman 2002; Solomon and Sibley 2002; Schnug et al. 2014). Bivalve molluscs, particularly MG and DP were chosen as they are (a) widespread, ecologically important representatives of both coastal and inland water bodies (Bayne 1976; McDonald et al. 1991; Binelli et al. 2015), (b) sessile, filter feeders capable of concentrating contaminants within their tissues, where in turn mussel health is closely related to the quality status of the aquatic environment to which they are found (Hawkins 1992; Souza et al. 2012) and (c) the physiology, anatomy and ecology of both species is well understood and their effectiveness within ecotoxicological studies well documented (Fig. 1.4).



Figure 1.4. *M. galloprovincialis* from Trebarwith Strand (Left) and *D. polymorpha* from Bude Canal, North Cornwall, UK (Right), and comparative external features and anatomy.

The anatomy of both species is comparable (Fig. 1.4); main components include shell valves, posterior/anterior muscle, gills, mantle, digestive organs, the foot and byssus. In terms of toxicity tests, haemocytes (cells circulating within open vascular system), gill and digestive gland cells are largely favoured. Two shell valves are relatively equal in size and held together via a large posterior or anterior adductor muscle. Suspension feeding and respiration occur via currents of water directed across the gills, food particles (i.e. algae, phytoplankton) are trapped by bands of lateral cilia on the gills and are directed to the mouth (Riisg et al. 2011). Filtration rates are dependent on environmental conditions, such as concentration of organic and inorganic particles and temperature (Riisg et al. 2011), close proximity to surrounding media makes gill a key organ of concern. Whilst mussels are generally sessile, movement is allowed via a large, muscular foot, it also serves as an anchor when stationary. Byssus threads, strong, proteinaceous fibres that originate from specialised glands within the foot (Silverman and Roberto 2010) allow the mussel to securely attach itself to a substrate.

The Mediterranean Blue mussel, MG is a marine bivalve found predominantly on rocky substrates of the intertidal and nearshore zones. The species has a broad latitudinal distribution that extends from the Mediterranean to parts of Australia and South America (McDonald et al. 1991). Its extensive biogeographic range is mainly attributed to tolerance to environmental variability. *Mytilus* spp. are of high ecological relevance; first, through the removal of particulates and excess nitrogen from the aquatic environment they improve water quality (Edebo et al. 2000; Petersen et al. 2014; Zhou et al. 2014). Second, mussel beds are often regarded to be ecosystem engineers. They provide a food source for many aquatic organisms and can offer habitats, along with nursery grounds for juvenile

fish and invertebrates (Crooks 2002; Gutiérrez et al. 2003; Borthagaray and Carranza 2007). MG are also cultured as a food source for human consumption, acting as an important protein source and means of income, linking them directly with human health.

In light of this the use of bivalves, such as MG and DP as bioindicator species for both monitoring and research purposes is extensive, a prominent example being the Mussel Watch Program (NOAA 2012). This contaminant monitoring program aims to actively research, assess and monitor the health status of estuarine and coastal environments via contaminant concentrations in bivalve tissue and sediments, providing effective, integrative ecosystem based management. *Mytilus* spp. are considered as surrogates for vertebrate models in laboratory based toxicity exposures (Matthiessen 2008). Individuals from the genus *Mytilus* have been used as sentinel organisms to monitor toxicity response to a range of contaminants, including radionuclides (Walker et al. 2000; Hagger et al. 2005; Jha et al. 2005, 6; AlAmri et al. 2012; Dallas et al. 2016a; Pearson et al. 2018), metals (Mohamed et al. 2014; Poynton et al. 2014; Lewis et al. 2016; Xu et al. 2016), pharmaceuticals (Schmidt et al. 2011; Gonzalez-Rey et al. 2014; Koutsogiannaki et al. 2014; Mezzelani et al. 2016) organic contaminants and engineered nanoparticles (Di et al. 2011, 17; Gomes et al. 2014a, b; Hu et al. 2015; Rocha et al. 2016). The effects of specific contaminants on MG can be determined on many biological levels (Table 1.2). Physiological responses such as mortality, clearance rate, growth and morbidity have been utilised as indicators of health, additionally subcellular and molecular markers. Biomarkers such as MN induction, DNA strand breaks, and more recently the addition of 'omics' technologies have provided an informative snapshot of the health status of a species within an environmental context.

DP, commonly known as the zebra mussel is a freshwater, invasive bivalve species. While originating in Russia their adaptable, tolerant nature, rapid growth and reproduction rate has allowed for their global distribution. Use as a freshwater counterpart for *Mytilus* spp. in ecotoxicological studies has been outlined by (Binelli et al. 2015).

Ubiquitous distribution, continuous availability throughout the year and ease of sampling has resulted in DP being an ideal bioindicator for freshwater environments, additionally high filtration rates result in high contaminant accumulation rates directly into tissue (Baldwin et al. 2002; Bervoets et al. 2005; Binelli et al. 2015). The rapid intake of toxicants allows for fast determination of negative biological effects, DP therefore act as an early-warning system of environmental stress (Binelli et al. 2015). DP is frequently used for biomonitoring of freshwater habitats such as lakes and rivers (Richman and Somers 2005; Riva et al. 2008; Voets et al. 2009; Alcaraz et al. 2011), and are utilised alongside *Mytilus* spp. in the NOAA's mussel watch contaminant monitoring program (NOAA 2012). In terms of ecological relevance, DP are an important food source for some aquatic invertebrates, fish, reptiles and birds, toxic substances may be transferred through the food web via consumption of contaminated individuals (Ghedotti et al. 1995; Tucker et al. 1996). Biomarkers including DNA alterations (DNA strand breaks or DNA adducts), MN formation and alterations in gene and protein expression have been found as reliable indicators of genotoxic and molecular level effects, caused by numerous contaminants in DP (Mersch and Beauvais 1997; Riva et al. 2011; Vincent-Hubert et al. 2011; Châtel et al. 2012, 2015; Parolini et al. 2015, 16; Magni et al. 2018). Combined utilisation of marine and freshwater species in this work will allow for determination of relative

sensitivity following IR exposure. To our knowledge, this is the first study to investigate IR-induced response in the freshwater mussel, DP.

1.5 Experimental design: Laboratory or field, and external factors

A growth in scientific knowledge in the field of radiobiology from both laboratory and field studies has been key in aiding radiobiological protection for both human, non-human biota and the environment (Brechignac and Doi 2009).

The principal advantage of biological toxicity tests performed in the laboratory is the ability to standardise methodologies. In terms of radiobiological studies it allows for a controlled exposure of an organism to a known radiation dose, providing direct evidence of IR as a causative agent of toxicity. Such controlled exposure conditions, especially when single species focused are replicable, reproducible, and easy to interpret (LaPoint et al 1989). Furthermore laboratory tests allow for comparison, this may be in highlighting the relative sensitivities of different aquatic organisms, or life history stages to a given contaminant, or ranking contaminants by relative toxicity (LaPoint et al 1989). The main disadvantage of such controlled exposures is the difficulties in extrapolating data to field conditions. Field studies offer the advantage of authenticity, they account for ecological (predator-prey relations, competition) or environmental (food resources, water quality, other contaminants) variation. However it is not possible to isolate a single causative factor. Toxicity may be related to the contaminant of interest, another environmental stressor (e.g. metals) or a combination of many, making results extremely difficult to interpret or replicate. Nuclear incidents, such as Chernobyl and Fukushima have provided scientists with a real world research site, allowing for the long term study of radiation induced biological effects in a range of biota.

In radiobiological terms, exposure is generally classed as acute or chronic. Acute exposures are often short and intense (typically hours or days), often referring to a large, single dose or series of doses for a short duration of time. Acute IR exposure can result from accidental release of radioactive material, from NPP incidents (i.e. Chernobyl or Fukushima) or weapons testing. While often lacking in environmental realism, acute toxicity tests can provide the basis for future, chronic exposures in terms of experimental dose range (Inc. LD₅₀), and the immediate MoA involved in IR-induced stress response (Paget and Barnes 1964; Walker et al. 2000). While such studies help to elucidate mechanisms of toxicity, they are only environmentally valuable where an accidental release of a contaminant occurs. Chronic exposure typically refers to continuous exposure (weeks, months, years) to low or environmentally realistic radiation levels over a long period of time. Chronic studies, whilst more time consuming, complex and expensive could be regarded as more relevant in displaying a more accurate representation of biological damage, as aquatic biota are typically exposed to contaminants over long durations of time, and over many generations (UNSCEAR 1996; Parisot et al. 2015). As some contaminants have differing MoAs under acute and chronic conditions it is vital to investigate biological damage under varying exposure setting.

1.6 Overall aims of research

In light of the above information, the main aims and objectives of this thesis are as follows:

1. Establish the effects of metal contaminant copper (Cu^{2+} , referred to as Cu throughout text) on marine and freshwater mussels using a suite of biological end points. This approach will also serve to validate our choice in biomarkers in both MG and DP gill cells for use in subsequent studies on radionuclides [Chapter 3];
2. Establish relative sensitivity between marine and freshwater bivalve species in response to IR and metals, alone and in combination [Chapters, 3, 4, 5 and 6];
3. Determine tissue specific accumulation, dose rate and depuration (release via excretion) of ^{32}P in two different species of mussel. Accumulation patterns will highlight key tissues of interest for future experiments investigating biological response. [Chapter 4];
4. Determine genotoxic and molecular responses in two bivalve species following ^{32}P exposure [Chapter 5];
5. Establish the relationship between accumulation and radiation dose to subsequent biological responses, in gill and digestive gland tissues [Chapter 5];
6. Determine the impact of Cu on the effects of ^{32}P exposure in two bivalve species [Chapter 6].

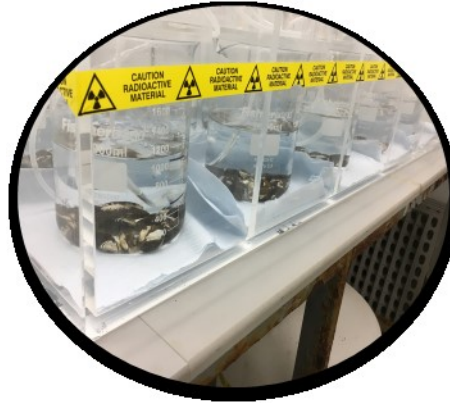
1.7 Hypothesis

1. Quantitatively and qualitatively, freshwater and marine mussels show comparative biological responses following exposure to metals and radionuclides, along with similar accumulation patterns (Cu and ^{32}P);
2. DP can be used as a freshwater representative to MG in ecotoxicological/radiation studies;
3. Tissue specific accumulation and dose rate could be correlated with biological responses in bivalves;
4. Bivalves could serve as sensitive indicators to assess biological responses following exposures to metals and radionuclides, either alone or in combinations.

The experimental chapters in this thesis (Including chapters 3, 4, 5 and 6) are written and formatted as papers for publication. Co-authors and journals are listed at the start of each experimental chapter where appropriate.

Chapter 2

Methods and method development



2.1 Chemicals and suppliers

All chemicals, reagents and consumables were purchased from Fisher Scientific Ltd, UK, Anachem Ltd, UK, Sigma-Aldrich Company Ltd, UK, VWR International Ltd, USA, Greiner Bio-One Ltd, UK, Perkin Elmer Inc., USA, LabLogic Systems Ltd, UK, Varicon Aqua Solutions Ltd, UK, Novus Biologicals LLC, USA, Bio-Rad Laboratories Ltd, UK, Bioline Reagents Limited, UK or AMS Biotechnology (Amsbio) Ltd, product details are noted in text as appropriate. Seawater was obtained from Plymouth Sound, stored on site, aerated, filtered (10 µM) and UV treated (VECTON UV water steriliser UV8, TMC, UK) before use.

2.2 Mussel collection and maintenance

Adult *D. polymorpha* (DP, 20-30 mm) and *Mytilus* sp. (45-60 mm) were collected by hand from Bude Canal, Cornwall, UK (latitude: 50 49' 41" N, longitude: 4 32' 58" W) and Trebarwith Strand, Cornwall, UK, as in Dallas et al (2013) and Vernon et al (2018) (latitude 50 38' 40" N, longitude 4 45' 44" W), respectively (Fig. 1.4., 2.1). DP were transported to the laboratory in sealed bottles (500 mL) containing canal water, and *Mytilus* sp. in sealed plastic bags, both stored on ice in a cool box (transportation < 2 h).

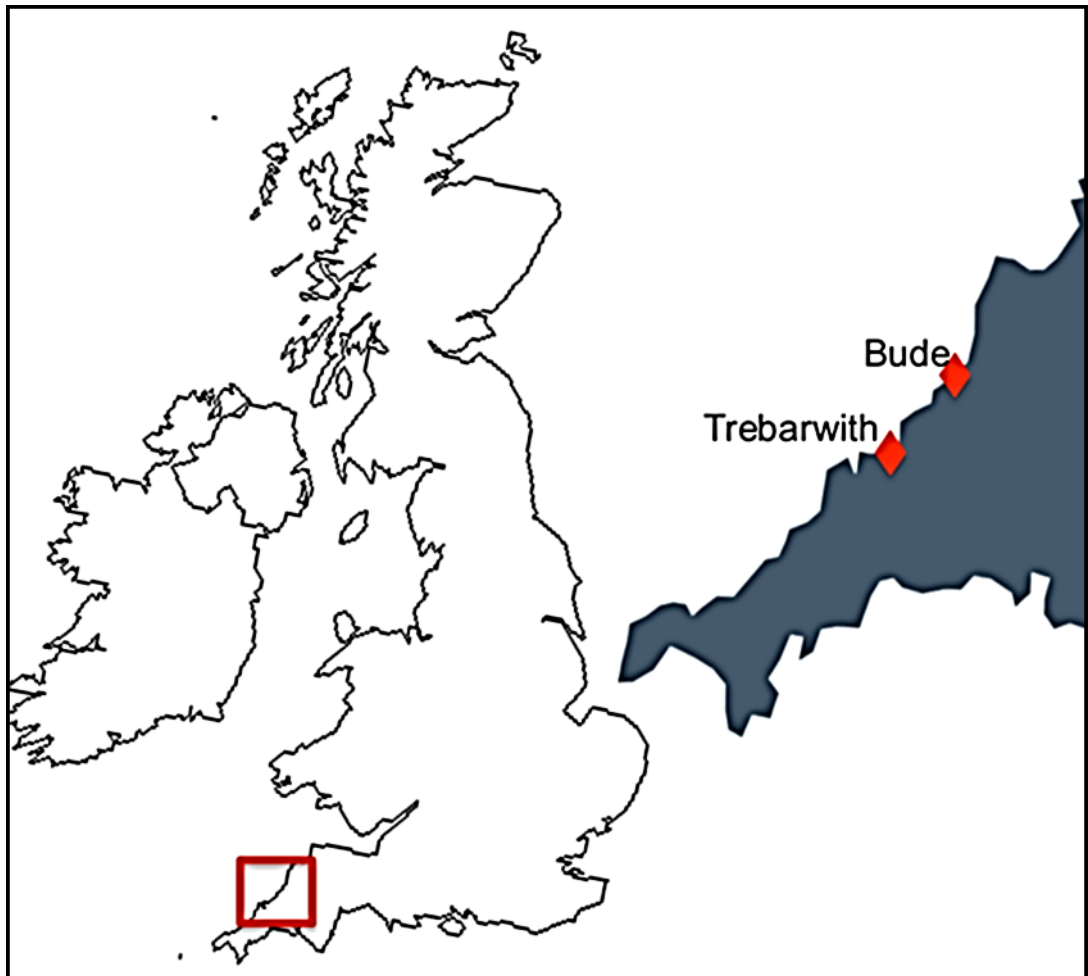


Figure 2.1. Map to show location of collection sites for *M. galloprovincialis* (Trebarwith, Cornwall) and *D. polymorpha* (Bude, Cornwall) mussels in the UK.

As in Dallas et al (2013), Dallas et al (2016a) and Vernon et al (2018), *Mytilus* sp. were transferred to a 75 L tank, filled with approximately 55 L of filtered ($< 10 \mu\text{m}$), UV treated, aerated seawater (~ 4.5 mussels L^{-1}). Both species were maintained at a 12:12 photoperiod at 15°C , in a temperature controlled room. As there was limited information on DP maintenance, there was a need to optimise this process before experimental use. To note, this was not necessary for *Mytilus* sp. due to extensive use within our laboratory and a well-established maintenance protocol. Cell viability (section 2.4) was used as indicator of mussel health; optimisation continued until individuals had a cellular viability above 80%. To assess health of DP in their natural habitat, cell viability in haemocytes extracted immediately after removal from Bude Canal were transferred to the laboratory on ice and analysed immediately (Fig. 2.2), cells were 87.6% viable on average.

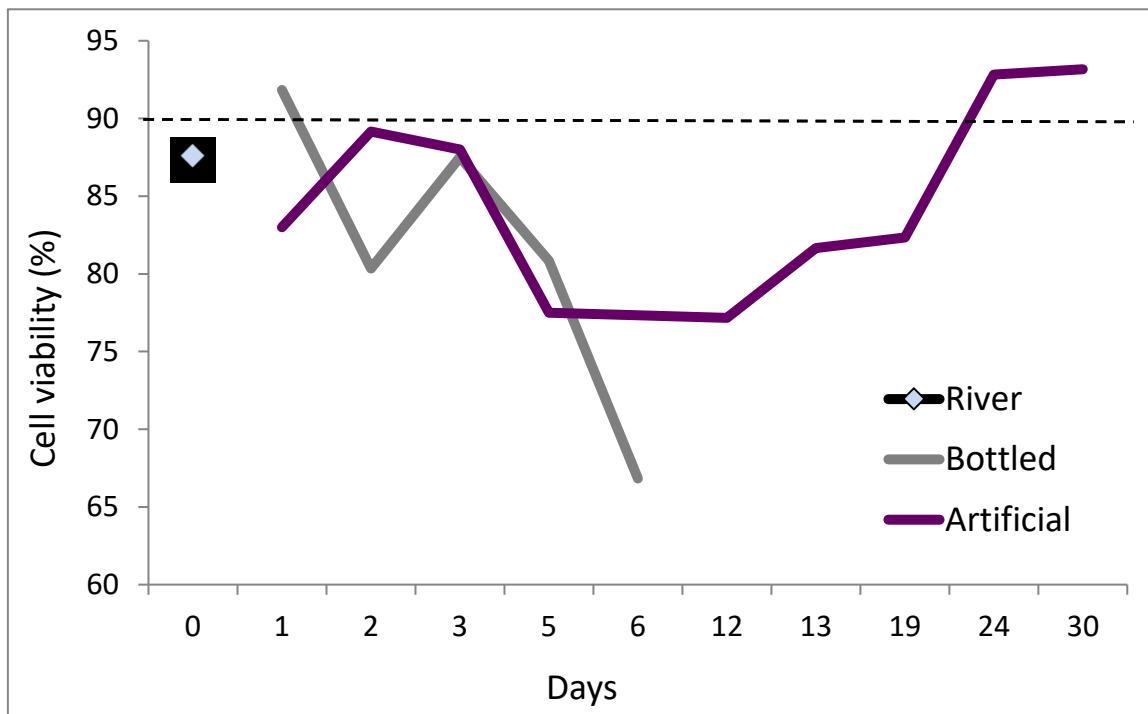


Figure 2.2. Cell viability (%) of *D. polymorpha*: Optimisation of water types (River, bottled and artificial) for maintenance in the laboratory ($n = 6$).

DP were initially kept in a 1 L plastic container in the following water types (a) tap water (main tap in Davy 422) and (b) bottled water (Highland Spring still water). Tap water was initially used as an inexpensive, easily available option, however it was not utilised due to possible variation of water quality with each water change (i.e. metal concentrations, pH). Bottled water was tested as a controlled, inexpensive choice. Cell viability was checked each day over 7 ± 3 days. On day 7, cell viability remained low at around 66.8 % ($n = 6$, bottled, Fig. 2.2). DP were then transferred to a 15 L aquarium, filled with 10 L aerated DI water (~ 5 mussels L^{-1}) and an artificial river water solution (2M $CaCl_2 \cdot 2H_2O$, 8 mM $MgSO_4 \cdot 7H_2O$, 40 mM, 5 mM KNO_3 , 0.7 M $NaHCO_3$). Water test kits (Tetra, Blacksburg, VA, USA) were used to assess and maintain optimum water quality parameters (carbonates, general hardness, nitrite, nitrates, chlorine and ammonia), along with dissolved oxygen (DO), salinity, pH and temperature (Hach HQ40D Multi-meter, Hach-Lange, Dusseldorf, Germany). Cell viability was maintained at 90 % ($n = 6$, Fig. 2.2).

During the holding period, mussels were fed three times per week. DP were fed on dried *Chlorella* powder (3.2 mg/mussel per feed, Naturya, Bath, UK), *Mytilus* sp. on a solution of *Isochrysis galbana* algae (8×10^5 cells mL^{-1} , Reed Mariculture, Campbell, CA, USA). A 70% (DP) and a 100% water change was performed ~ 2 h after feeding. Mussels were allowed a 2-week acclimatisation period before use in experiments. Due to the invasive nature of DP (Nalepa and Schloesser 1992; Karatayev et al. 2007), all wastewater was spiked with salt ($50 g L^{-1}$, NaCl) before disposal as to prevent infestation and dispersal.

2.2.1 Genetic identification of *Mytilus* spp.

Hilbish et al (2002) reported the presence and distribution of *Mytilus edulis*, *M. galloprovincialis* and their hybrids in the coastal regions of southwest England. Based only on morphological characteristics alone these species are very difficult, if not impossible to differentiate (Koehn 1991; McDonald et al. 1991). Small genetic variations may account for differential biological response, particularly at molecular level (Hilbish et al. 1994). Species homogeneity in our experiments was therefore ensured based on the method of Inoue et al (1995). In accordance to Dallas et al (2016a), Dallas et al (2018) and Pearson et al (2018), species at the collection site were confirmed as *M. galloprovincialis* (MG). To note, this was not necessary for DP.

A small section of gill tissue (~25 mg) was dissected from each individual ($n = 20$, main collection site), snap frozen immediately in liquid nitrogen, and stored at -80°C until analysis. Tissue was homogenised, Chelex solution added (500 μl , Bio-rad, 10%), samples vortexed and incubated (56°C , 30 min, vortex every 10 min). Following incubation, samples were boiled (98°C , 8-10 min) and placed on ice for 3 min. Samples were then spun (5 min, max speed $\sim 32,310\text{ g}$), and had supernatant removed (in duplicate). Samples were stored (4°C short term, -20°C long term) until analysis.

Diagnostic Me 15 and Me 16 PCR primers (Fig. 2.3) were used to analyse the genetic composition of species. These validated markers, which target the adhesive protein gene sequence (Glu-5' gene, GenBank accession no. D63778) have successfully been utilised to identify the morphologically similar species (Inoue et al. 1995; Coghlan and Gosling 2007; Dias et al. 2008; Kijewski et al. 2011; Pearson et al. 2018). Length of amplified fragments vary interspecifically at 180, 126 and 168 base pairs for *M. edulis*, *M. galloprovincialis* and *M. trossulus*

respectively, individuals with both bands are considered as hybrids (Inoue et al. 1995).

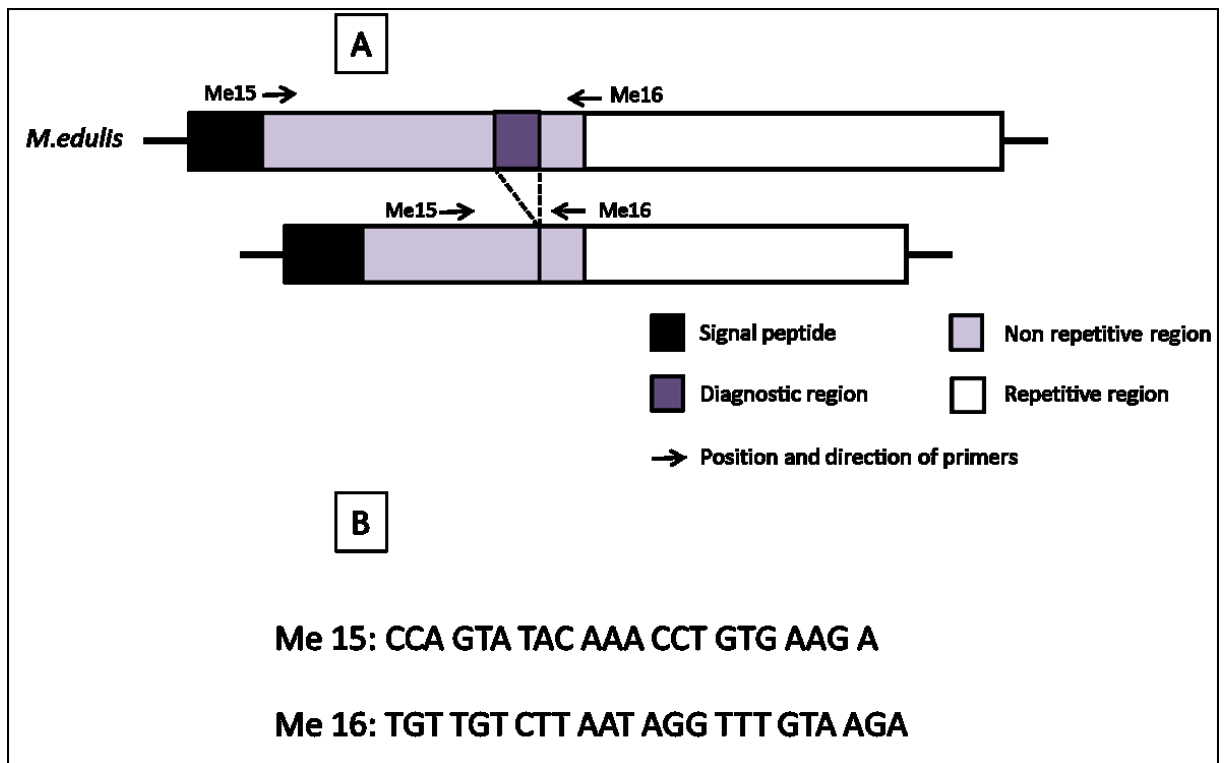


Figure 2.3. Genetic identification of the *Mytilus* spp. (A) Positions of PCR primers in the Glu-5' gene in the *M. edulis* and *M. galloprovincialis* and (B) sequences of the primers (modified from Inoue et al (1995)).

Standard PCR amplification was carried out; first reagents (20 µL molecular grade water, 25 µL myTaq red mix, 1 µL primers and 3 µL sample DNA) were combined and vortexed briefly. Samples were spun (10 s, max speed ~ 32,310 g) and transferred into a thermocycler (Applied Biosystems). A cycling profile of 94 °C (initial denaturation), 30 cycles of 94 °C, 30 s (denaturation), 56 °C, 30 s (annealing of primers), 70 °C, 1.5 s (extension, taq activity), followed by a 72 °C, 5 min final extension was performed. Samples were stored at -20 °C until analysis.

For running PCR products on a TBE gel, a gel tray was filled with a 1x TBE (80 mL, Tris borate EDTA buffer), 2 % agarose (1.6 g, molecular grade, bioline) and SYBR safe (2 µL, 10,000x stock) solution, left at room temperature (45 min) to set. Once in the electrophoresis tank, 15 µL of ladder and 10 µL sample is placed into individual wells, along with a negative control (no DNA sample added). Gels were run at 90 V for approximately 1 h, and imaged using the Image-quant LAS 4000 (Fig. 2.4).

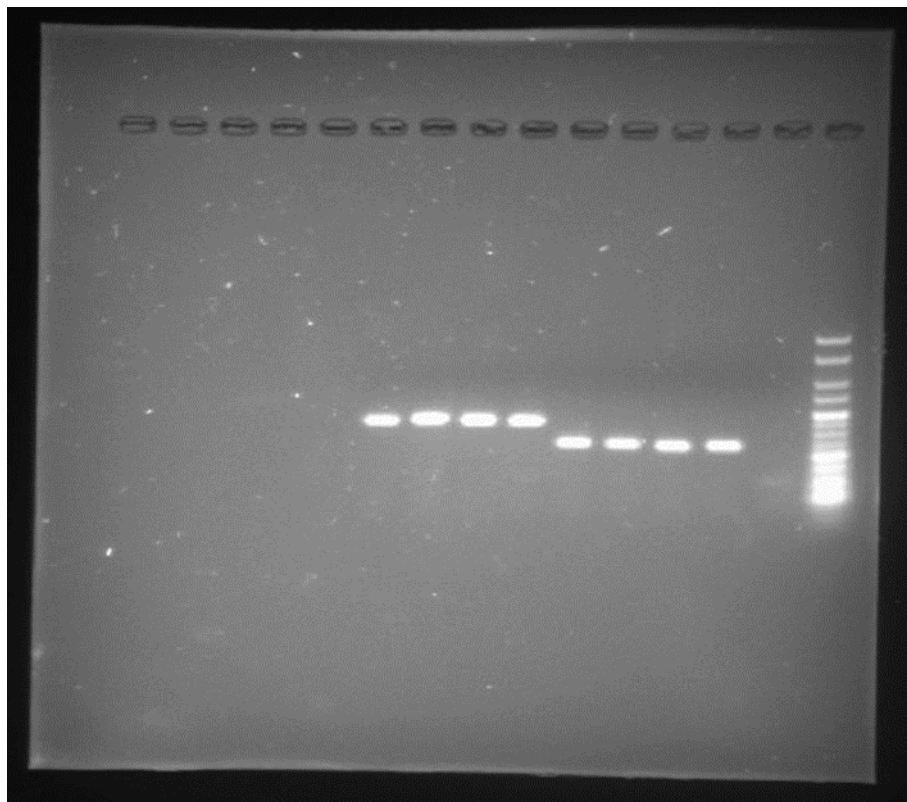


Figure 2.4. Me 15/16 PCR products visualised on agarose gel (*Mytilus* spp.) (Image: L. Dallas, 2014) (A) *Mytilus edulis* and (B) *Mytilus galloprovincialis*

2.3 Cellular extractions for biological assays

2.3.1 Haemolymph extraction

Extraction methods were comparable for both the species. Haemolymph was extracted by gently opening the mussel shell using a small pair of scissors, which were also used to hold the shell open by about 2-3 mm (Fig. 2.5). A 21 and 25-gauge needle, attached to a 1 mL syringe was used for the MG and DP, respectively (both needles BD Microlance, Fisher Scientific Ltd, UK). The needle was inserted carefully into the adductor muscle (Fig. 1.4, 2.5). A successful extraction from MG would be approximately 1 mL, 0.4 mL for DP (Fig. 2.5). Haemolymph was stored in a microcentrifuge tube on ice, in the dark before experimental use.

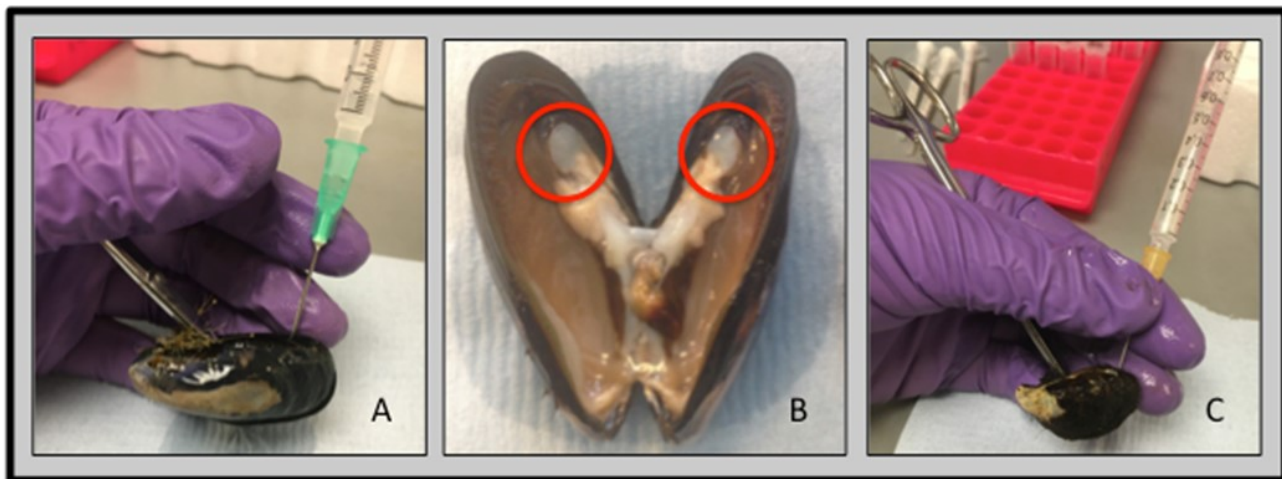


Figure 2.5. Haemolymph extraction from adductor muscle (B, red circles) in: *M. galloprovincialis* (A) and *D. polymorpha* (C).

2.3.2 Tissue digestion

For assays requiring a single cell suspension (i.e. comet assay, MN assay, gamma-H2AX [γ -H2AX]) a tissue digest was performed, the procedure was adapted from earlier studies (Vincent-Hubert et al. 2011), with minor adjustments. Following dissection under reduced light, a small section of tissue (e.g. gill, digestive gland cells) was stored in a microcentrifuge tube on ice. Dispase II solution (1 mL total volume, 1.6 mg dispase powder per 1 mL of HBSS, Sigma-Aldrich Company Ltd, UK) was added per sample. Samples were incubated at 37 °C, and shaken every 10 min (30 min total time). Following incubation, cell suspension was removed (leaving any large lumps) and centrifuged at 160 g for 5 min. The supernatant was then used in subsequent assays. This technique was successfully adapted for the gill, digestive gland, mantle and posterior adductor muscle cells of MG and DP (Fig. 2.6).

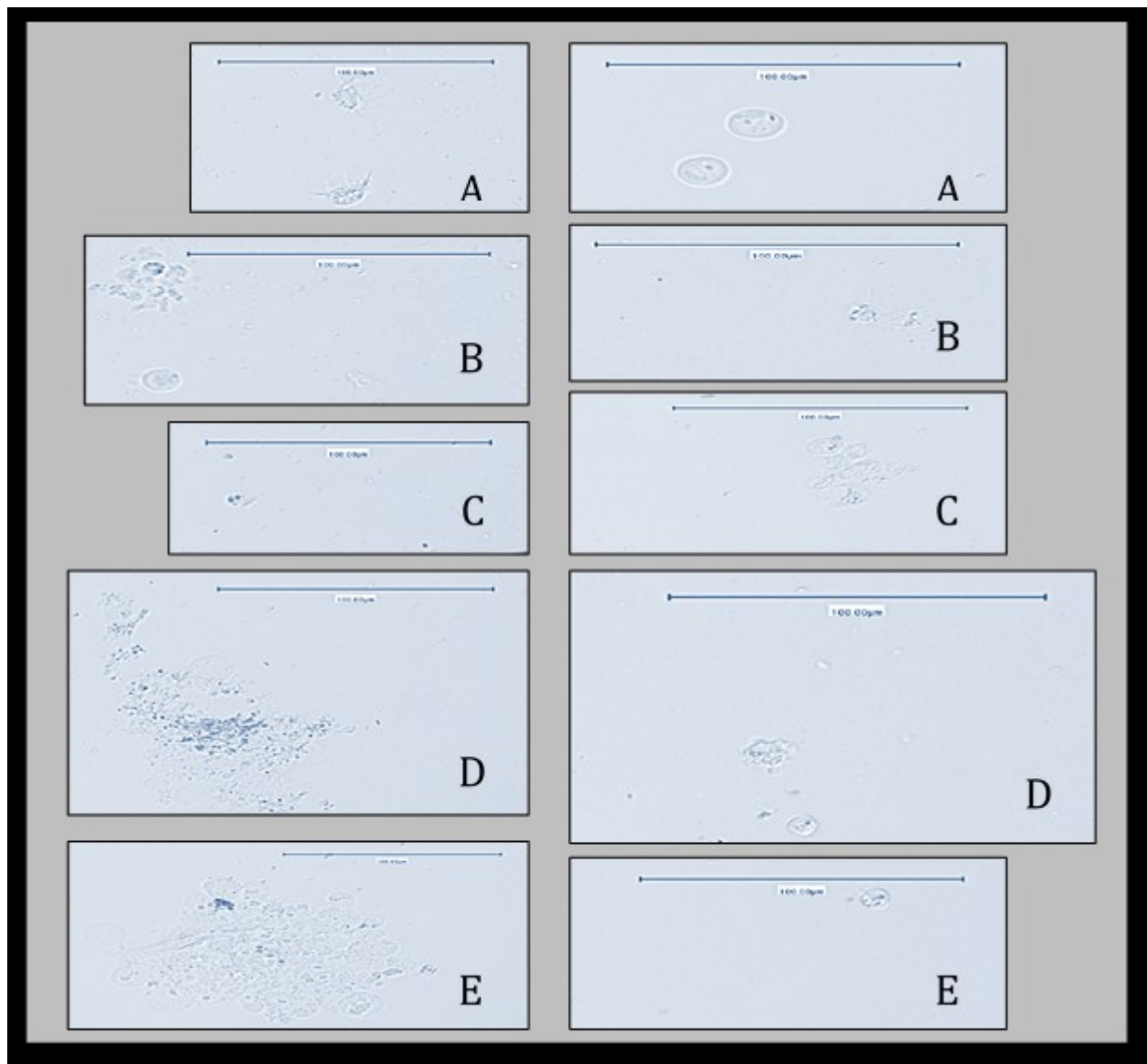


Figure 2.6. Different cell types in *M. galloprovincialis* (left) and *D. polymorpha* (right): Haemocytes (A) gill cells (B) digestive gland cells (C) mantle cells and (D) posterior adductor muscle cells (E) (100 µm scale).

2.4 Cell viability assessment with Trypan blue

Viability of mussel haemocytes, gill and digestive gland cells was assessed using the Trypan Blue exclusion dye assay (Strober 2001). Live cells have intact cell membranes that exclude certain dyes, whereas nonviable, dead cells are left with a blue colouration (Fig. 2.7). A 10 µL subsample of single cell suspension was transferred to a microcentrifuge tube and gently mixed with 1 µL Trypan Blue

(0.4%, Sigma), the solution was then transferred to a slide, and a coverslip was applied. The number of viable cells (clear cytoplasm), and dead cells (blue cytoplasm) were examined under light microscopy (x 40). Samples with >90% viability were used in subsequent tests.

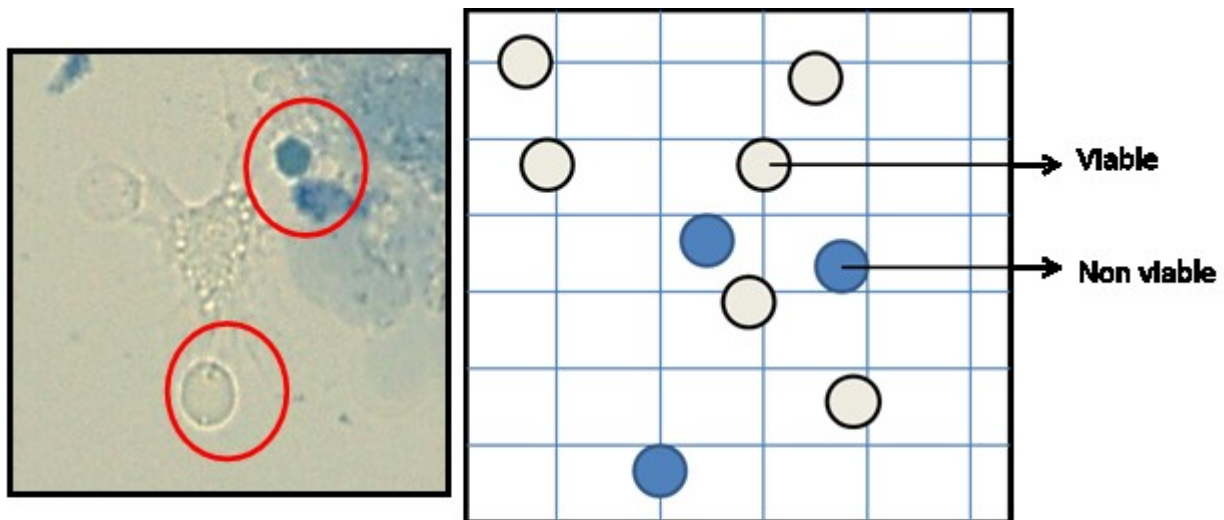


Figure 2.7. Cell viability in *M. galloprovincialis* haemocytes using the Trypan blue exclusion method. Red circles surround viable (clear) and non-viable (blue cells).

2.5 Biological assays

2.5.1 Micronucleus (MN) assay

The MN assay is a simple, reliable cytogenetic assay, which determines the presence of micronuclei in the cytoplasm of interphase cells (Bolognesi and Fenech 2012). MN formation is indicative of DNA damage or mutation, formation mainly originates from either a whole chromosome, chromosome fragment containing centromere or from acentric chromosome fragments (Fig. 2.8). The assay detects the activity of clastogenic (chromosome breakage) and aneugenic agents (abnormality in chromosome number within daughter cell). This assay can be performed using mussel haemocytes or tissue cells, it has been successfully

utilised in a wide range of aquatic invertebrates such as bivalves (Bolognesi et al. 2004; Jha et al. 2005; Jha et al. 2006; Bolognesi and Fenech 2012; Chandurvelan et al. 2013; Dallas et al. 2013; Michel et al. 2013; D'costa et al. 2018), crustaceans (de la Sienna et al. 2003; Barka et al. 2016) and fish (Cavas 2011; Omar et al. 2012; Kumar et al. 2014; Orozco-Hernández et al. 2018) to a wide range of environmental contaminants.

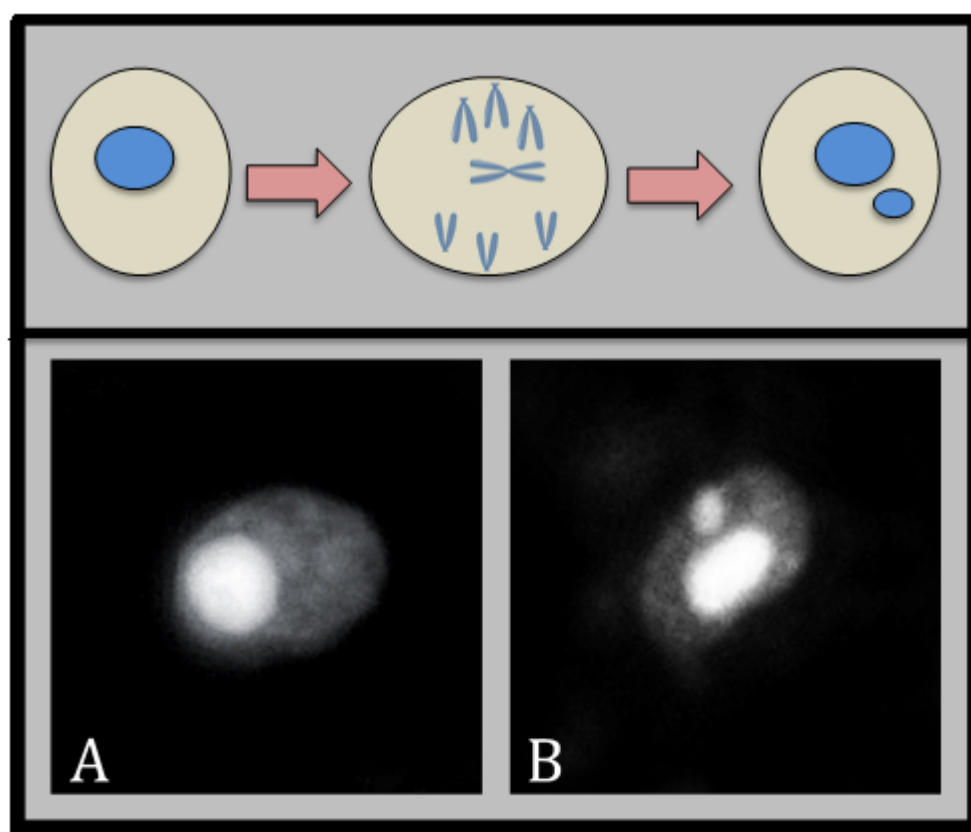


Figure 2.8. Simplified micronuclei (MN) formation (top). Normal cell (A) cell with micronuclei (B) (bottom). Both cells are *M. galloprovincialis* gill cells stained with ethidium bromide.

The procedure to prepare slides for the analysis of MN was adopted as described elsewhere in detail (Bolognesi and Fenech 2012; Dallas et al. 2013), with minor modifications. Cell suspension (haemocytes or tissue digest, 50 μ L) was smeared onto a coded slide (1 h, 4 $^{\circ}$ C) to allow cells to adhere. For fixation, ice-cold

carnoy's solution (1 mL, 75% methanol, 25% glacial acetic acid) was gently pipetted onto slide and left for 20 min. Fixative was carefully tipped off, slides were rinsed gently with DI water, and allowed to dry overnight (room temp). To score, slides were stained with 20 μ L ethidium bromide (20 μ L of 20,000 μ g L⁻¹). In total, 500 cells were scored per slide using a fluorescent microscope (DMR; Leica Microsystems, Milton Keynes, UK), slides were scored at random to prevent bias. MN classification was in accordance to Venier et al (1997) and Bolognesi and Fenech (2012). Results are reported as mean MN per 1000 cells, in keeping with other data from our research group (Dallas et al. 2013).

2.5.2 Comet assay

The comet assay (single cell gel electrophoresis assay [SGCE]/microgel electrophoresis assay) is used to detect single/double strand breaks (SSB/DSB) in individual cells, it is regarded as a relatively simple, sensitive technique (Collins 2004; Jha 2008). Additionally it offers rapid, reproducible results without the need for cell division, as an alternative to cytogenetic assays (Jha 2008). This technique, adopted by the Organisation for Economic Co-operation and Development (OECD) to test chemicals in the human health arena, has been utilised in a wide range of aquatic organisms of differing life history stages. Taking bivalves as an example, it has proved successful in highlighting the genotoxic effect of radionuclides (Hagger et al. 2005; Jha et al. 2005, 6; Simon et al. 2011; Kumar et al. 2014; Dallas et al. 2016a; Pearson et al. 2018), metals (Guidi et al. 2010; Al-Subiai et al. 2011; Trevisan et al. 2011; Vosloo et al. 2012), pharmaceuticals (Canty et al. 2009; Petridis et al. 2009; Parolini et al. 2011a) and nanoparticles (Canesi et al. 2014; Girardello et al. 2016; Koehl  -Divo et al. 2018).

This assay can be performed on haemocytes or tissue cells, as described in sections 2.3.1 and 2.3.2. Cell suspension (150 μL) from each individual is used immediately for the comet assay. A cell-agarose suspension (1% LMA, Sigma-Aldrich Company Ltd., UK) was pipetted in duplicate onto a pre-coated (1% NMA in TAE) slide and left to set (4 $^{\circ}\text{C}$, ~ 1 h). Slides were then placed in a chilled lysis buffer (1 h, 4 $^{\circ}\text{C}$) and denatured in an electrophoresis buffer (20 min, 0.3 M NaOH and 1 mM EDTA, at pH 13). Electrophoresis was run for 25 min at 21 V and 620 mA. Slides were placed in a neutralisation buffer (0.4 M Tris Base, Sigma) for 5 min and then distilled water for another 5. DNA is stained with ethidium bromide (20 $\mu\text{g mL}^{-1}$, working solution) and examined using an epifluorescent microscope (DMR; Leica Microsystems, Milton Keynes, UK). One hundred cells per slide (50 cells per gel) were quantified using Comet IV imaging software (Perceptive Imaging, Bury St Edmunds, UK) software (Fig. 2.9). The software provides results for different parameters, % Tail DNA was considered the most reliable to present the results (Kumaravel and Jha 2006).

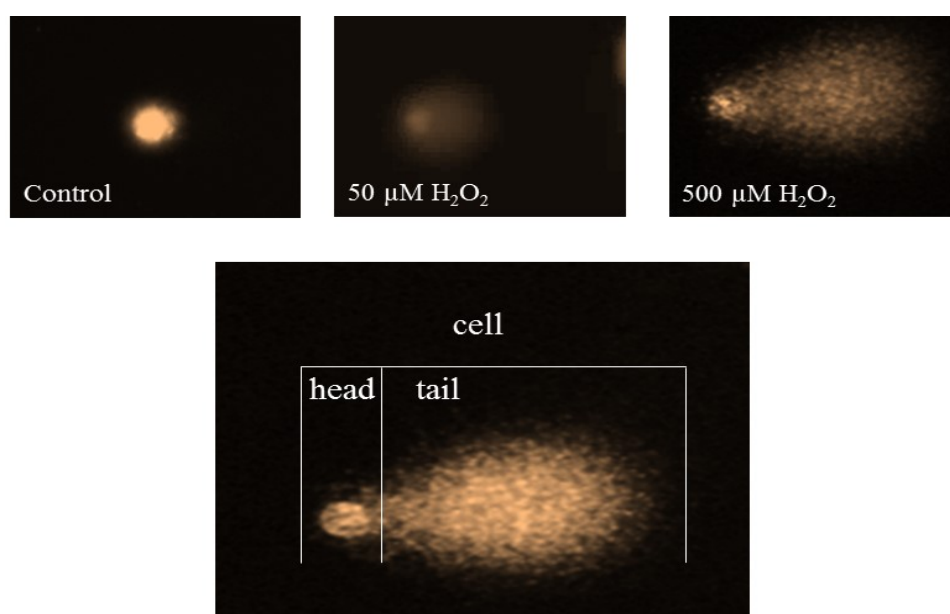


Figure 2.9. Scoring comet assay slides: *M. galloprovincialis* gill cells: Varying levels of DNA damage in response to hydrogen peroxide exposure (top), head and tail of comet (bottom).

2.5.2.1 Optimisation and validation of the comet assay using hydrogen peroxide (H₂O₂)

After a 2-week depuration period, gill, digestive gland tissue and haemolymph was extracted as outlined in sections 2.3.2 and 2.3.1 respectively from MG and DP ($n = 4$). Gill and digestive gland tissue was digested to acquire a single cell suspension, cell suspensions were pooled to reduce inter-individual variability and stored on ice in the dark until use. Aliquots (150 μ L) of cells were transferred into microcentrifuge tubes and spun (775 g, 2 min, 4 °C). Once supernatant was removed and discarded (leaving approx. 10 μ L), a H₂O₂ solution (Sigma H1009, 8.8 M, 100 μ L, 0, 5, 50 or 500 μ M in PBS) was added to the cellular pellet. H₂O₂ was used for validation as a known genotoxic agent, it has been successfully utilised to validate genetic biomarkers in previous literature (Dallas et al. 2013; Sarkar et al. 2015). Following incubation (1 h, 4 °C, dark) the samples were spun (as before), supernatant removed, and samples processed as in section 2.5.2. In both species, H₂O₂ concentrations produced an increase in percentage tail DNA in comparison to the control (Fig. 2.10, $p < 0.01$ and $p < 0.01$ in haemolymph, $p < 0.01$ and $p < 0.01$ in gill and $p < 0.001$ and $p < 0.001$ in digestive gland, in DP and MG).

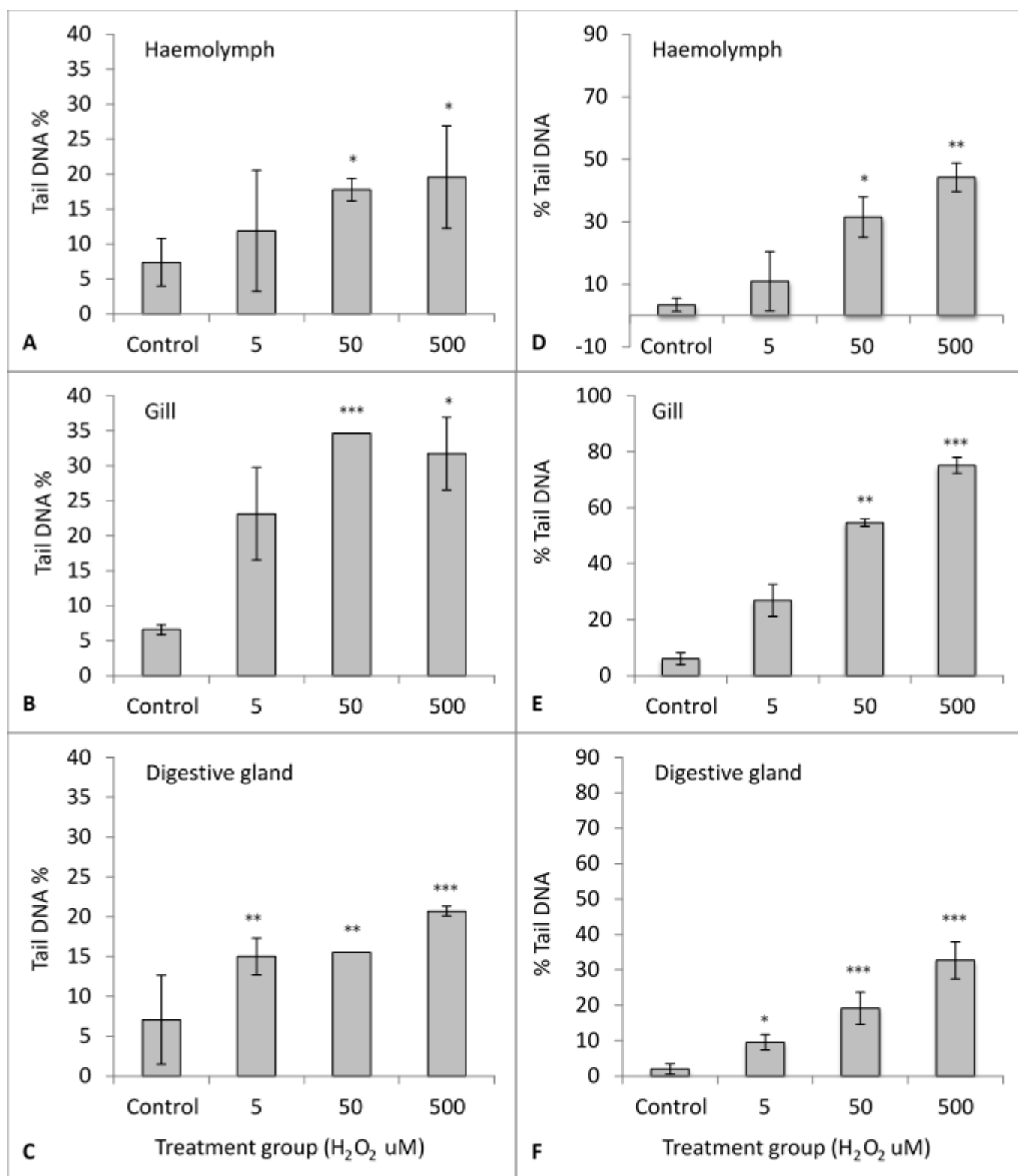


Figure 2.10. DNA damage (% Tail DNA) in *M. galloprovincialis* (A, B and C) and *D. polymorpha* (D, E and F) haemocytes, gill and digestive gland cells, following exposure to varying concentrations of hydrogen peroxide. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01 , 0.001) from the corresponding control. SD is standard deviation of mean data. $n = 4$.

2.5.3 53BP1 and γ -H2AX assays

DSBs are a detrimental form of DNA damage for the cell. To prevent mutations that could subsequently result in genome instability, it is essential that damage is detected and repaired before DNA replication and cell division (FitzGerald et al. 2009). γ -H2AX and Tp53-binding protein 1 (53BP1) play an important role in DNA DSB checkpoint activation and repair (Fig. 2.11).

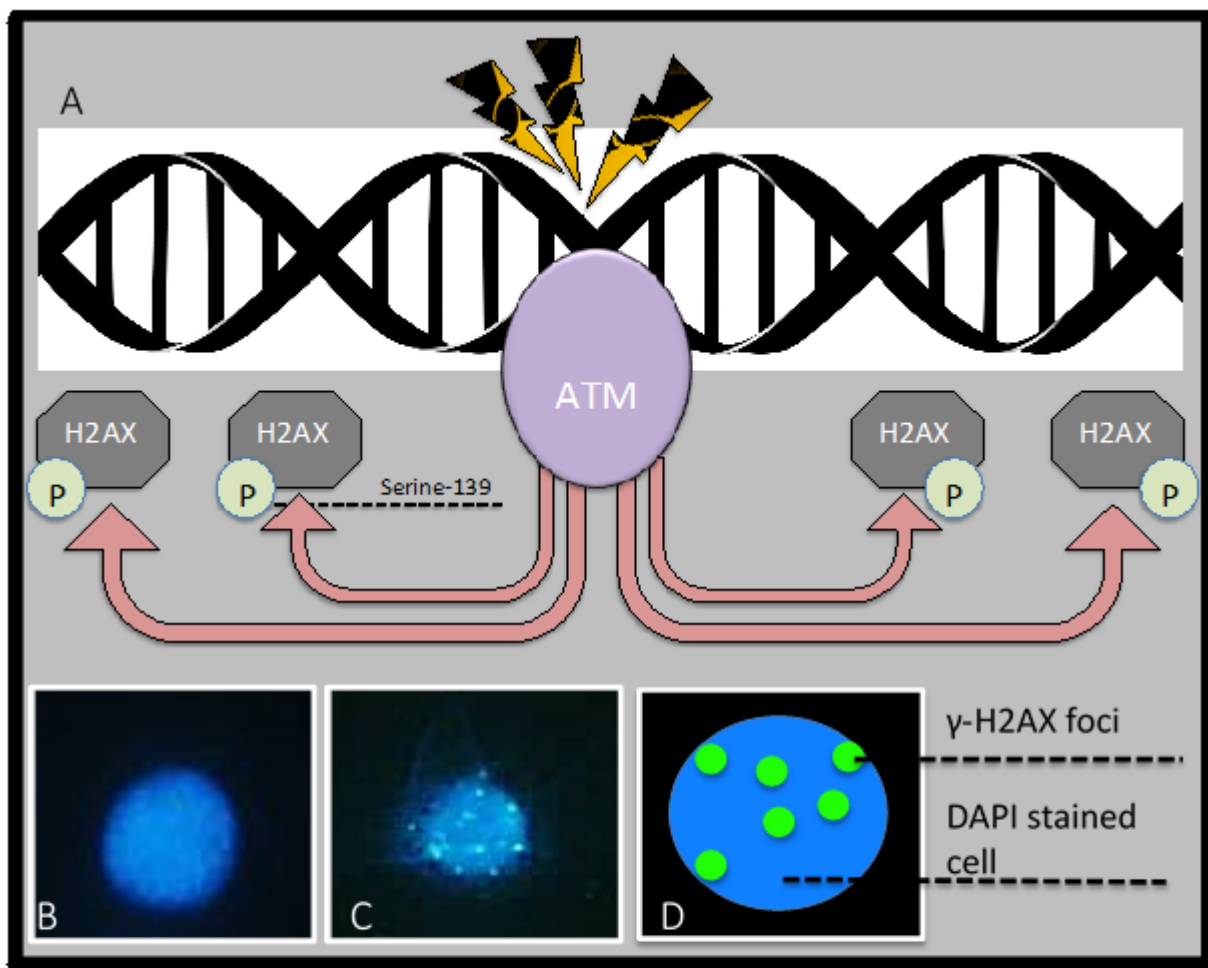


Figure 2.11. Diagram illustrating the recruitment of phosphorylated H2AX following ATM activation (A). Bottom Images show: Healthy cell stained with DAPI (B), DAPI stained cell with foci induction (C), and illustration showing cell and green foci (D) (images B and C: *D. polymorpha* gill cells).

Following a DSB, MRN-mediated ATM activation (including ataxia telangiectasia mutated [ATM], ataxia telangiectasia and rad3 related [ATR] and DNAPK [DNA-dependent protein kinase]), initiate a series of phosphorylation events. γ -H2AX, the phosphorylation of the histone H2A variant H2AX at Ser-139 is thought to act as a hold or site for the recruitment of proteins necessary during repair of DSBs (Ozaki et al. 2014). γ -H2AX appears rapidly and forms foci at damage sites, and DSBs are represented in a 1:1 manner (Kuo and Yang 2008). Being highly conserved from yeast, to zebrafish ZF4 cells, to humans (Foster and Downs 2005; Liu et al. 2011; Pereira et al. 2011; Sayed et al. 2017), we were able to utilise antibodies from differing species to measure γ -H2AX foci induction in marine and freshwater bivalves (González-Romero et al. 2012). Although not fully investigated to date in aquatic invertebrates, González-Romero et al (2012) presented the first study identifying the existence of functionally differentiated histones H2A.X and H2A.Z in the chromatin of MG individuals.

53BP1 is of interest as one of the proteins promoted to damaged chromatin, where it then promotes non-homologous end-joining-mediated DSB repair (Panier and Boulton 2014). 53BP1 has been well studied in humans and has been found to regulate the choice between DSB repair pathways, promoting non-homologous end-joining (NHEJ) and inhibiting homology-directed repair (HDR), additionally it binds to and promotes the mobility of damaged chromatin (Zimmermann and de Lange 2014). Due to a lack of sequencing data it is not yet known whether this protein is conserved in aquatic bivalves, however preliminary studies in MG and DP suggest a dose dependant response to in vitro H₂O₂ exposure in haemocytes and gill cells.

The technique for γ -H2AX and 53BP1 only varies in terms of antibody used, γ -H2AX response was confirmed in multiple tissue cell types (in both MG and DP) using standardized techniques in our laboratory on malignant human cells (Oommen et al. 2016a; Oommen et al. 2016b) (Fig. 2.12). Tissue cells (over haemocytes) were favoured for three reasons. Firstly, the DP mussels' small size (relative to MG species) allowed for a limited volume (0.4 μ L approx.) of haemolymph to be extracted, when carrying out a multi-biomarker study a greater volume is required. Secondly, haemocytes can be separated into sub-groups by morphological and biochemical properties, composition and type may vary between species or individuals, resulting in potential variations in biological response (Hine 1999). Lastly, haemocytes circulate within an open vascular system (Delaporte et al. 2003) in close contact with epithelial cells and the outside environment. Whilst also true with gill cells there is more background noise, or 'debris' in the haemolymph samples. Background noise can be eliminated somewhat via a PBS rinse (see below for details) or chelation, however this dramatically reduced the cell count.

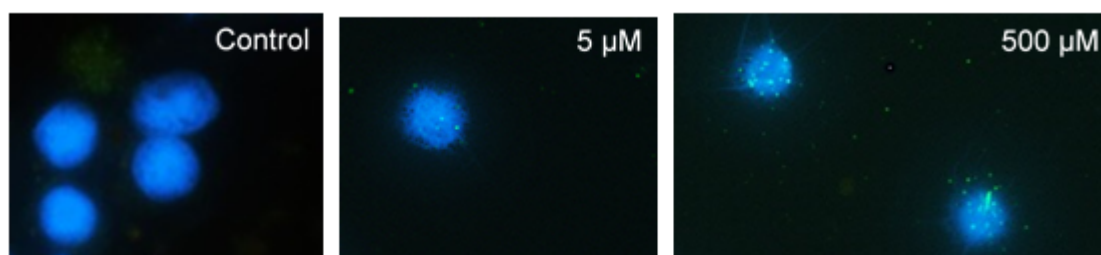
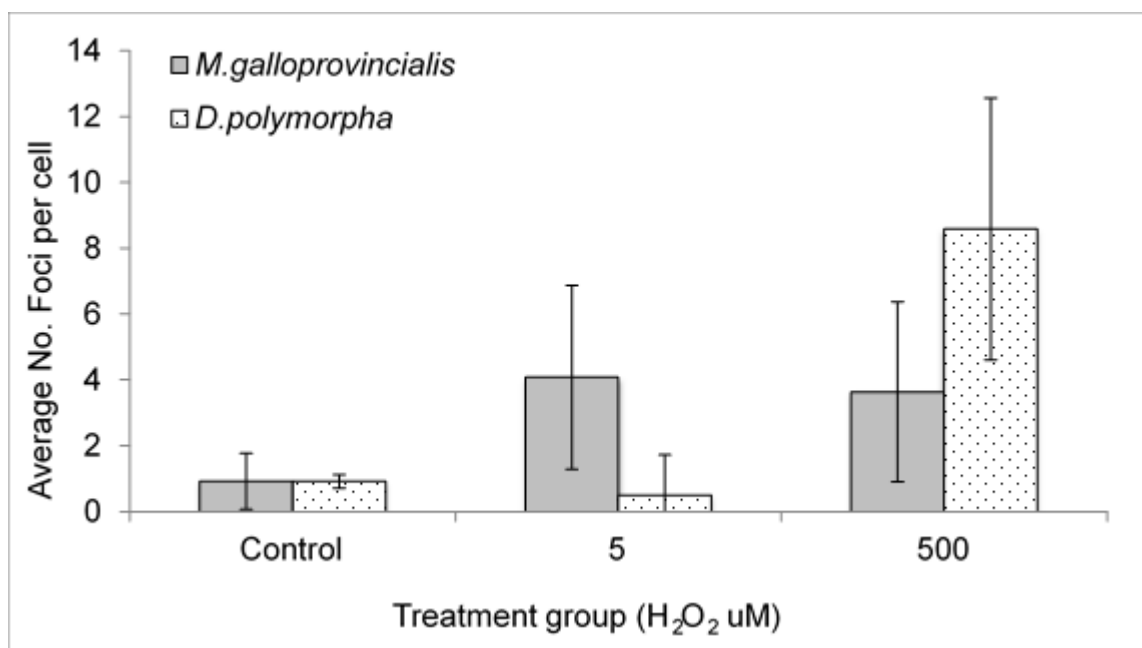


Figure 2.12. Average number of γ -H2AX foci in *M. galloprovincialis* and *D. polymorpha* gill cells following exposure to varying concentrations of hydrogen peroxide for 1 h. SD is standard deviation of mean data. Images show induction of γ -H2AX-foci in *D. polymorpha* gill cells. $n = 4$.

Two methods were tested for cell fixation. Firstly cell suspension (100 μ L) was smeared onto a slide and chilled in the fridge for 1 h. Slides were tilted gently to remove excess liquid. Due to a low cell count (after running complete assay) we tried fixation method two. Microscope slides with coverslips gently rested into place were positioned into appropriate slots in a cytocentrifuge (Cytospin™ 4, Fisher Scientific UK Ltd). To note, both coverslips and slides were used with and

without an IMS rinse, this did not appear to affect results. Special attention was made to ensure that filter, slide and coverslip were in line with each other, and that the hole in the filter was in a proper position so that cells were able to reach the slide. Cell suspensions (50 μ L) were aliquoted into the appropriate wells of the cytopsin, which was subsequently run at 140 g, 5 min. Coverslips were carefully removed and placed in individual wells of a multiwell plate (6 well plate, sterile, Greiner Bio-One, UK). Slides were chilled (20 min, 4 °C) to allow adhering of cells and fixed with ice-cold Carnoys solution (20 min, 4 °C, 1 mL per well). Subsequent to fixation, coverslips were rinsed with PBS (Dulbecco, Fisher, UK) in triplicate. Three additional slides were performed each experiment as a control. To allow entry of cell-impermeable fluorescent probes, cells were permeabilised for 10 min (0.5% Triton X-100 in PBS, room temp). To prevent the non-specific binding of the antibodies, cells were blocked for 1.5 h with normal goat serum (60 μ L per coverslip, G9023, SIGMA). Alternative blocks included foetal bovine serum (FBS, 10% FBS in 0.1% Triton X-100/PBS), bovine serum albumin (BSA, 3% BSA in 0.1% Triton X-100/PBS) and milk powder (Marvel dried milk powder [MP], 5% MP in 0.1% Triton X-100/PBS), for varying lengths of time (1, 4.5 and 13 h, Fig. 2.13). Cells were then rinsed in triplicate with 0.1% Triton X-100/PBS. Primary antibody was optimised at varying lengths of time (1, 4 and 13 h) under different temperatures (room temperature and 4 °C). Following incubation (overnight, 4 °C) with the primary antibody (60 μ L per slide, 1:10000 in 0.1% Triton X-100/PBS, anti-GamaH2H [γ -H2AX]), cells were rinsed (in triplicate, 0.1% Triton X-100/PBS) and incubated in the dark with the secondary antibody (1 h, 60 μ L per slides, 1:1000 in 0.1% Triton X-100/PBS, Anti-IgG secondary antibody, room temp). The slides were then rinsed as before. Procedural blanks were run alongside samples, with no primary antibody.

Cells were counterstained with DAPI (1 μg 10 mL^{-1} PBS, 10 min in dark) and rinsed (in duplicate, DI water). Coverslips were gently removed from well plates, tilted to remove excess liquid and mounted onto labelled slides. Slides were scored using a fluorescence microscope (NIKON Epifluorescence 80i, 60x magnification), by counting the number of foci in each of 50 cells per individual/slide. As described by Festarini et al (2015), cell nuclei were located with an appropriate DAPI filter, and a FITC filter set for the FITC signal of the γ -H2AX primary antibody. All coded slides, including procedural blanks were scored 'blind'.

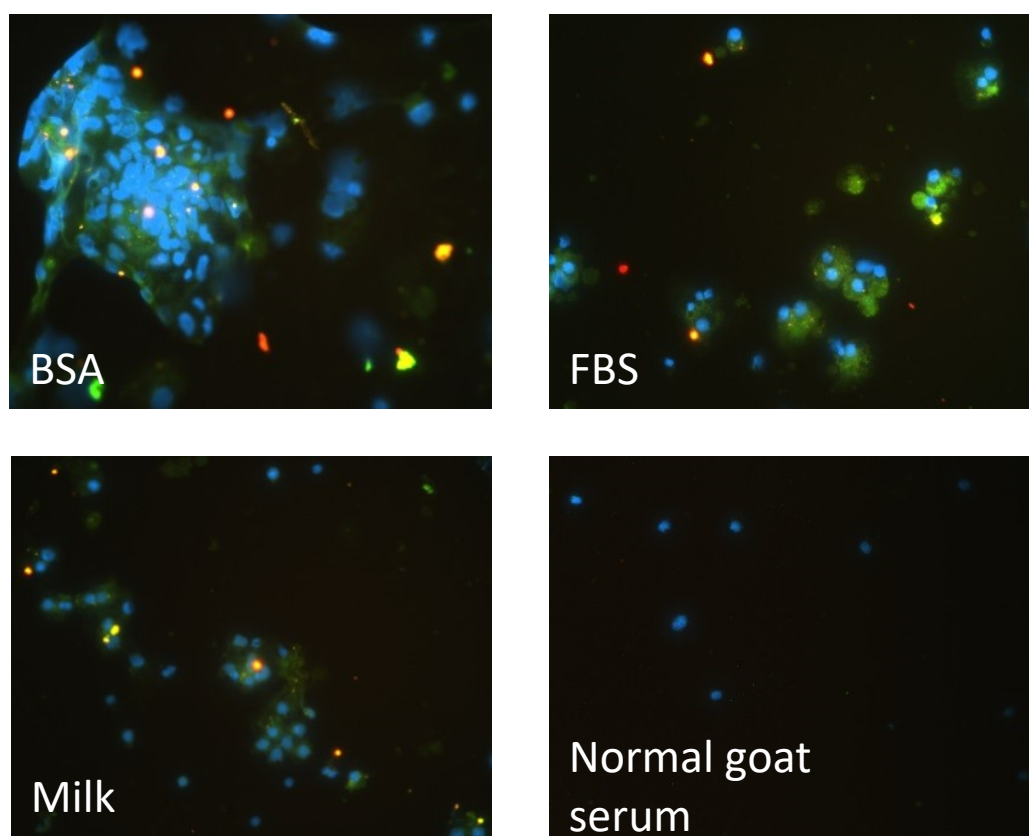


Figure 2.13. Optimisation of γ -H2AX assay. Images show microscope image of DAPI stained *M. galloprovincialis* gill cells, subsequent to varying blocks (Bovine serum albumin [BSA], foetal bovine serum [FBS], milk powder and normal goat serum).

2.5.3.1 Validation of the γ -H2AX assay using H_2O_2 as a reference agent

Prior to Cu exposure, γ -H2AX response was optimised and validated under *in vitro* conditions in numerous cells, including haemocytes, gill and digestive gland cells of both the bivalve species using standardized techniques on human cells in our laboratory conditions (Oommen et al. 2016a, b). Validation was performed using H_2O_2 (0, 5, or 500 μM in PBS). In accordance with section 2.5.3, a single gill cell suspension was collected ($n = 4$) and pooled to reduce inter-individual variability, samples were stored on ice until use. Samples were exposed to H_2O_2 as in section 2.5.2.1. The protocol was then run as standard (see section 2.5.3). In both species, H_2O_2 concentrations produced an increase in percentage foci per cell in comparison to the control ($p = 0.01$ and $p = 0.04$) for DP and MG, respectively (Fig. 2.14).

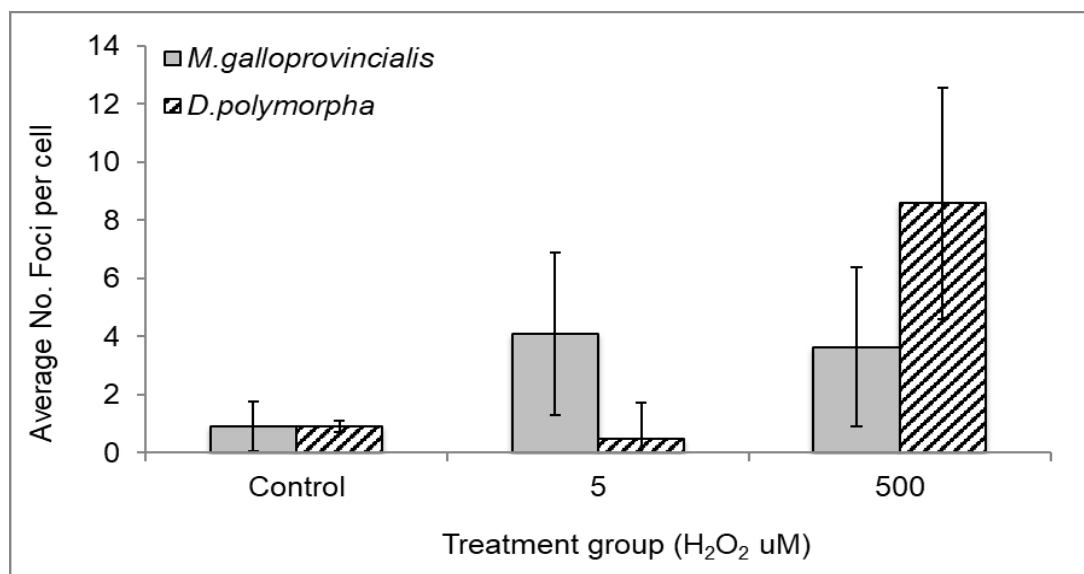


Figure 2.14. Average number of γ -H2AX foci in *M. galloprovincialis* and *D. polymorpha* gill cells following exposure to varying concentrations of hydrogen peroxide. SD is standard deviation of mean data. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. $n = 4$.

2.5.4 Transcriptional expression of key genes

DNA is the primary molecule of concern for effects from IR and genotoxic chemicals, expression of key genes can provide an early signal of organism stress. The following criteria were set when choosing genes of interest, (a) for means of comparison, well documented markers of oxidative and stress responses in both study species (preferably in both gill and digestive gland tissue) and (b) available primer sequences. The genes (including primer sequences) selected for this study have been listed in Table 2.1. These include superoxide dismutase (*sod*), catalase (*cat*), glutathione S-transferase (*gst*), and heat shock proteins 70 (*hsp70*) and 90 (*hsp90*). In accordance with Navarro et al (2011), Lacroix et al (2014), Dallas et al (2016a) and Banni et al (2017), elongation factor 1 (*ef1*) and β -Actin (*act*) were chosen as reliable, widely utilised housekeeping genes for both study species.

Table 2.1. Summary of primer pairs used for reference and target genes in *M. galloprovincialis* and *D. polymorpha*: Name, abbreviation, primer sequences and accession number.

Gene	Short name	Forward primer	Reverse primer	GenBank Accession No.
<i>Mytilus galloprovincialis</i>				
Actin	act	5'-GGGAGTCATGGTTGGTATGG-3'	5'-TCAGAAAGGACTGGGTGCTCT-3'	AF157491
Elongation factor 1	ef1	5'-CACACGAGTCTCTCCAGA-3'	5'-GCTGTCAACACAGACCATTC-3'	AF063420
Glutathione S-transferase	GST	5'-ATGGCTCTTTCCCTGCTTCA-3'	5'-TTTGGCCAGTGCCCATGTTA-3'	AF527010
Superoxide dismutase	CuZn-sod	5'-TGCAGGATCACATTCAACCCA-3'	5'-TGTCCTGCTTAGCTCATGGCCA-3'	AJ581746
Catalase	CAT	5'-CACACGAGTGCTCTTCTGTT-3'	5'-CTTCCGAGATGGCGTTGTAT-3'	AY743716
Heat-shock Protein 70	HSP70	5'-GGGTGGTGGAACCTTTTGATG-3'	5'-GCCGTTGAAAAAGTCCTGAA-3'	AF172607
Heat-shock Protein 90	HSP90	5'-TCAGTGATGATCCTAGATTAGGCA-3'	5'-CGTTCTCTCTTCCATCTGTAAC-3'	AJ625655
<i>Dreissena polymorpha</i>				
Actin	act	5'-CCTCACCCCTCAAGTACCCCAT-3'	5'-TTGGCCCTTTGGGTGAGTG-3'	AF082863
Elongation factor 1	ef1	5'-CCACCAAGGAGCCCAAGAG-3'	5'-TGGGACGAGGTCA GCCATAC-3'	AJ250733
Glutathione S-transferase	GST	5'-TCCGCTATATCGCCTGGAC-3'	5'-GCTCCTTCAGACCTGCTTTC-3'	EF194203
Superoxide dismutase	SOD	5'-GACAGCATGGCTTCCATGTG-3'	5'-AGATTCTGGGCCAGTCAGAG-3'	AY377970
Catalase	CAT	5'-ACGGCTATGGAAGCCACACG-3'	5'-AGGTCGCGCATCGCATAGTC-3'	EF681763
Heat-shock Protein 70	HSP70	5'-TGTCCTGCTTGGAATGTAG-3'	5'-CGTGGTGAATGCTGTGTAG-3'	EF526096
Heat-shock Protein 90	HSP90	5'-TTGATCATCAATACTTCTATTG-3'	5'-ACACCAACTGTCCAATCAT-3'	GU433881a

2.5.4.1 RNA extraction

Gill and digestive gland tissue was dissected immediately after exposure and stored in RNAlater at -80 °C (R0901, Sigma-Aldrich Company Ltd UK) until use. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (RTN350, Sigma-Aldrich Company Ltd UK). To release RNA, biological tissue, lysis buffer (guanidine thiocyanate and 2-mercaptoethanol) and glass beads (2 mm, Sigma-Aldrich) were transferred into a microcentrifuge tube, and homogenised (FastPrep®, 30 s, 5 m/s) to form a smooth lysate (Fig. 2.15, A). Lysates were transferred into a filtration column and spun (2 min, ~19000 g), removing impurities such as cellular debris (Fig. 2.15, B). Subsequent to the addition of 70% ethanol (Ethanol, Absolute 200 Proof, Fisher Scientific), the filtrate was spun (2 min, ~19000 g) in a nucleic acid binding column (Fig. 2.15, C). To remove impurities such as protein and salt residues, two wash steps were carried out followed by a dry spin to ensure a clean eluent. Lastly, RNA was released from the binding column via an elution buffer. The concentration and purity of isolated RNA was determined by UV spectroscopy (Nanodrop 3300, Thermo Scientific, Fig. 2.15, D), total RNA was stored at -80 °C.

2.5.4.2 cDNA synthesis

cDNA was synthesised using the Applied Biosystems High-Capacity cDNA reverse transcription kit (Fisher Scientific, UK) with RNase inhibitor. A 10 µL RNA sample (1 µg RNA) and 10 µL RT master mix (10X RT buffer, 25X dNTP mix, 10X RT random primers, MultiScribe™ reverse transcriptase, RNA inhibitor) was added per tube (Fig. 2.15, E, F). Samples were sealed, spun to remove air bubbles (~19000 g, 10 s) and placed in the thermal cycler (Applied Biosystems) under the following conditions: 10 min, 25 °C; 120 min, 37°C; 5 min, 85 °C and

held at 4 °C. cDNA samples were diluted 1:5 with molecular water before qPCR (Fig. 2.15, G).

2.5.4.3 Real-time Polymerase chain reaction (qPCR)

cDNA (1.9 µL), PowerUp™ SYBR™ Green Master Mix (7.5 µL), forward/reverse primers (0.03 µL/primer) and molecular grade water (5 µL) were added to a 96 well plate (MicroAmp™ Fast Optical 96-Well Reaction Plate, 0.1 mL) to make a total volume of 15 µL per well (Fig. 15, H). All samples were run in duplicate. Plates were sealed (MicroAmp™ Optical Adhesive Film), spun and placed in the qPCR (Applied Biosystems Step-One Plus real-time PCR system with StepOne Software (v2.2.2; Applied BioSystems) under the following conditions: 2 min, 95 °C; 40 cycles of 95 °C for 15 s and 1 min, 60 °C (Fig. 2.15, I). A disassociation curve (melt curve) was run to verify the consistency of the PCR products (Fig. 2.15, J).

2.5.4.4 Data analysis and interpretation

In accordance with Dallas (2016a, 2018), relative mRNA expression ratio (RER) of key genes (*sod*, *cat*, *gst*, *hsp70* and *hsp90*) was quantified using REST (v 1, 2009), from PCR efficiencies calculated using LinReg PCR software (LinRegPCR version 2017.1) (Ramakers et al. 2003; Ruijter et al. 2009) and threshold cycle (C_q , Fig. 2.15, K, L). Values were normalised to the geometric mean of C_q determined for reference genes actin (*act*) and elongation factor 1 (*ef1*), using control samples to calibrate.



Figure 2.15. Diagram illustrating stages of gene expression protocol

2.6 Physiological observations

2.6.2 Byssal attachment and valve activity

Byssal attachment of individual mussels was assessed every alternate day of exposure by eye (Chapter 6, Fig. 2.16A), as an indicator of mussel health.

A primary response of bivalve molluscs when exposed to waterborne contaminants is to close their shell/valves as a form of avoidance behaviour (Kramer et al. 1989; Hartmann et al. 2015), by doing so reducing contaminant uptake. Valve movement or activity (i.e. whether the individual was actively filtering or had a fully closed shell) was adopted as a non-invasive technique to measure physiological variations in response to IR and metal over the duration

of the exposure (Chapter 6). Individuals were assessed by eye, three times daily for valve activity (Fig. 2.16B, C).

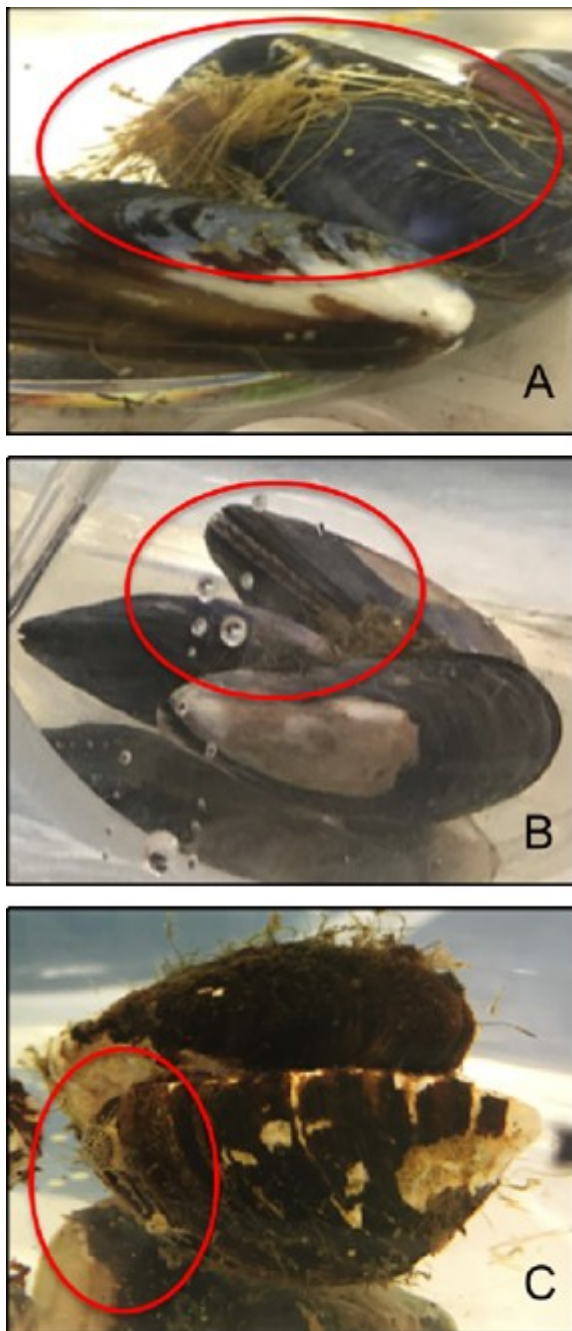


Figure 2.16. (A) Byssus thread produced by *M. galloprovincialis*, (B) *M. galloprovincialis* filter feeding and (C) *D. polymorpha* filter feeding

2.7 Determination of metal concentration using ICP-OES and ICP-MS

2.7.1 Tissue preparation

For validation of biological assays, copper (Cu^{2+}), as a metal toxicant and reference agent was utilised. Determination of Cu in water and tissue samples was carried out using ICP-MS (Inductively coupled plasma mass spectrometry) as described before in publications from our laboratory (Al-Subiai et al. 2011; Dallas et al. 2013; D'Agata et al. 2014). Briefly, all glass/plastic-ware and dissection tools were acid washed (2% nitric acid, Fisher Scientific) at least 24 h prior to use. Tissue was dissected and transferred into pre-weighed falcon tubes, samples were placed in an oven to dry ($60\text{ }^{\circ}\text{C}$, typically $> 48\text{ h}$). Tubes were re-weighed and recorded for dry weight. For tissue digestion, 1 mL (volume dependant on tissue weight, typically 2-3 mL for 0.1 g up to 10 mL for 1 g) concentrated nitric acid (N/2272/PB17, Fisher Scientific) was added to each tube. As procedural blanks, 6 tubes were tested alongside containing 1 mL acid without tissue, along with a certified reference material ($n = 3$, TORT-2 Lobster hepatopancreas, NRCC). Samples were boiled for 2 h (water bath, $80\text{ }^{\circ}\text{C}$), or until tissue was fully digested. Once cool, digests were diluted with 4 mL Milli-Q water and analysed as in section 2.7.2.

2.7.2 Determination of metal in water and tissue samples

To determine Cu concentration in water, samples (1 mL) were spiked with 50 μL hydrochloric acid immediately after extraction, and stored at room temperature until analysis. As in Dallas et al (2013), indium (^{115}In) and iridium (^{193}Ir) were used as internal standards in both tissue and water samples. Seawater samples were diluted 1:5 with DI water. Appropriate Cu standards were made to calibrate

the instrument before and during analysis of samples, in accordance to Al-Subiai et al (2011). Using appropriate parameters (^{63}Cu and ^{65}Cu), standards and samples were run using X Series II ICP-MS (Plasma Quad PQ2 Turbo, Thermo Elemental, Winsford, UK) with PQ Vision 4.1.2 software. Procedural blanks were run every 10 samples.

2.8 Ionising Radiation

2.8.1 Selection of radionuclide and determination of radiation dose levels

^{32}P (1.709 MeV, Table 2.2), along with its relative low cost and ease to work with was chosen as a study radionuclide as it can be used as a proxy for beta and gamma emitters, such as environmentally relevant ^{137}Cs and ^{90}Sr . In addition, its short half-life at 14.29 days is beneficial in terms of waste disposal. All waste material, such as unused ^{32}P solution, bivalve remains and general laboratory waste (i.e. pipette tips, gloves) can be stored for a relatively short time period of (approximately 4 months), before disposal. As illustrated in Table 2.3, several other radionuclides were investigated for use in IR studies, however ^{32}P was deemed the most suitable. In terms of suitability of alternative radionuclides iodine-131 had a half-life (8 d) too short to be utilised during experiments, both ^{33}P and tritium lacked the decay energy to be used as proxies for gamma emitters and $^{137}\text{Cs}/^{90}\text{Sr}$ were far more stringent in terms of safety regulations and allowed dose levels in the laboratory. Experimental dose rates of 0.10, 1 and 10 mGy d⁻¹ were based around a screening no-effect dose of 10 µGy h⁻¹ (0.24 mGy d⁻¹) (Garnier-Laplace and Gilbin 2006; Andersson et al. 2008, 9; Garnier-Laplace et al. 2008).

Table 2.2. Table outlining properties of phosphorus-32.

Property	³² P, P-32
Neutrons	17
Protons	15
Isotopic mass	31.973907274
Half-life (t _{1/2} ; d)	14.29
Decay mode	Beta, β-
Decay energy (MeV)	1.709
Decay products	³² S
Decay equation	$^{32}_{15}\text{P} \rightarrow ^{32}_{16}\text{S}^{1+} + e^{-} + \bar{\nu}_e$
Natural abundance	Trace
Maximum range in air (m)	6
Maximum range in water (m)	0.008

Table 2.3. Alternative radionuclides considered for experimental design.

Radionuclide	Short name	Decay mode	Decay energy (MeV)	Half-life	Decay products
Phosphorus-32	³² P	Beta	1.709	14.29 d	³² S
Phosphorus-33	³³ P	Beta	0.25	25.3 d	³³ S
Tritium	³ H	Beta	0.01859	12.32 y	³ He
Caesium-137	¹³⁷ Cs	Beta/gamma	(β-) 0.5120 (γ) 0.6617	30.17 y	^{137m} Ba
Strontium-90	⁹⁰ Sr	Beta	0.546	28.79 y	⁹⁰ Y
Iodine-131	¹³¹ I	Beta	0.971	8 d	¹³¹ Xe

2.8.2 Radiation safety and experimental design

All shielding and protective measures (Fig. 2.17 A-D) were purchased (Trent Plastics Ltd, UK) and put into place before any radioactive experiments were carried out, along with a hazard map detailing all areas of which radioactive materials would be present. Swab tests were carried out after each exposure.

To check for leaks, the shielding was filled with DI water and a fluorescent dye (Fluorescein, Fisher Scientific UK, 50 mg L⁻¹), and left for one week (Fig. 2.17E), no leaks were observed. A 10-day mock exposure was performed in September 2016 to test safety procedures outlined in the local rules and to practice routine procedures (Fig. 2.17E). Glass beakers (2 L total volume, 1.6 individuals L⁻¹) were placed in individual shielded compartments, containing 1.8 L filtered seawater (<10 µm) or freshwater. To determine possible contamination routes, fluorescein was added per beaker (dilution as above). The exposure was carried out in accordance with the local rules (see appendices), using the fluorescein solution in replacement of ³²P. Following the exposure, the experimental room and equipment's used were inspected with a UV torch (Vansky, 395nm) to determine any areas of contamination, none was observed.

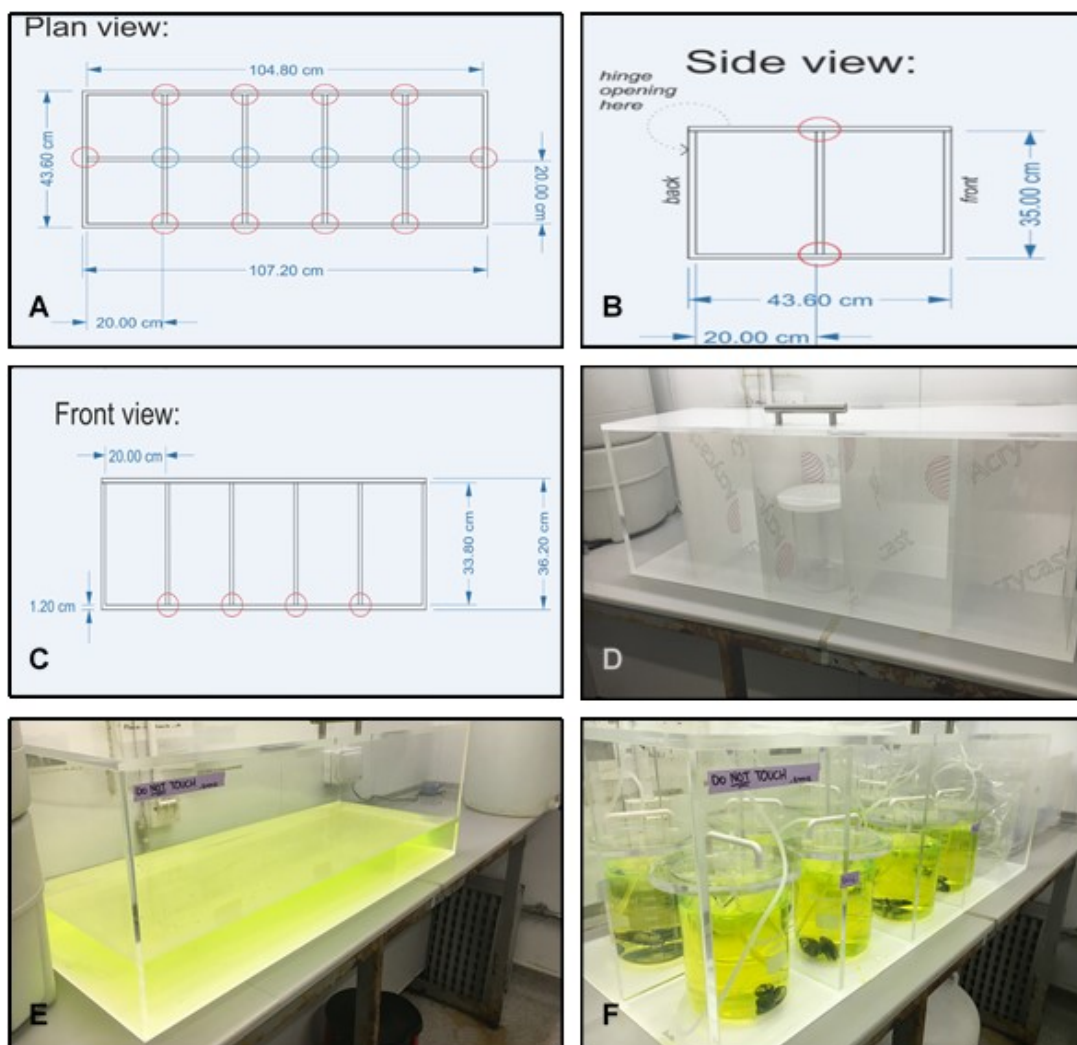


Figure 2.17. ^{32}P shielding: Designs and measurements for shielding (A-C), shielding with beaker covered with acrylic lid in individual compartment (D), safety exposure using fluorescein as surrogate for ^{32}P (E-F).

2.8.3 Liquid scintillation counting (LSC)

2.8.3.1 Tissue preparation for LSC

Two methods were tested for tissue preparation for LSC (Fig. 2.18) as there is no established technique for measuring ^{32}P concentrations in mussel tissue, the first method was adopted from previous protocols from our laboratory. In method one, tissue samples were transferred to pre-weighed scintillation vials (Fisherbrand™ Borosilicate Glass), freeze-dried (< 24 h, or until pressure is constant at 50-60 μbar) and weighed (to obtain dry weight). Samples were

rehydrated with 100 μL mQ water, 1 mL of Soluene-350 (Perkin Elmer) is then transferred into each vial. Samples are incubated at 55 $^{\circ}\text{C}$ for 48 h, to aid solubilisation samples were shaken (vibroMax) at 200 rpm. To bleach samples, 100 μL (in duplicate) H_2O_2 (8.8 M) was added, samples were left at room temperature for 30 min. To degrade H_2O_2 and stop reaction, samples were incubated at 60 $^{\circ}\text{C}$, 30 min. Subsequent to bleaching, all samples were mixed thoroughly with 5 mL scintillation cocktail (UltimaGold, Perkin Elmer) and 100 μL glacial acetic acid, left in the dark for at least 2 h prior to counting in a LS 6500 liquid scintillation counter (Beckman Coulter Inc., Brea, CA, USA) to a fixed precision of 5%. Radioactivity was determined by counting each sample for 2 h. Data was converted to Bq kg^{-1} using the dry weight of the tissue samples and to a dose rate in $\mu\text{Gy h}^{-1}$ using wet weights and the ERICA tool. Due to (a) long tissue processing time, (b) lack of sensitivity in measuring low ^{32}P levels using the Beckman Coulter LSC and (c) long sampling times (e.g. ~ 2 h per sample), this method was not utilised.

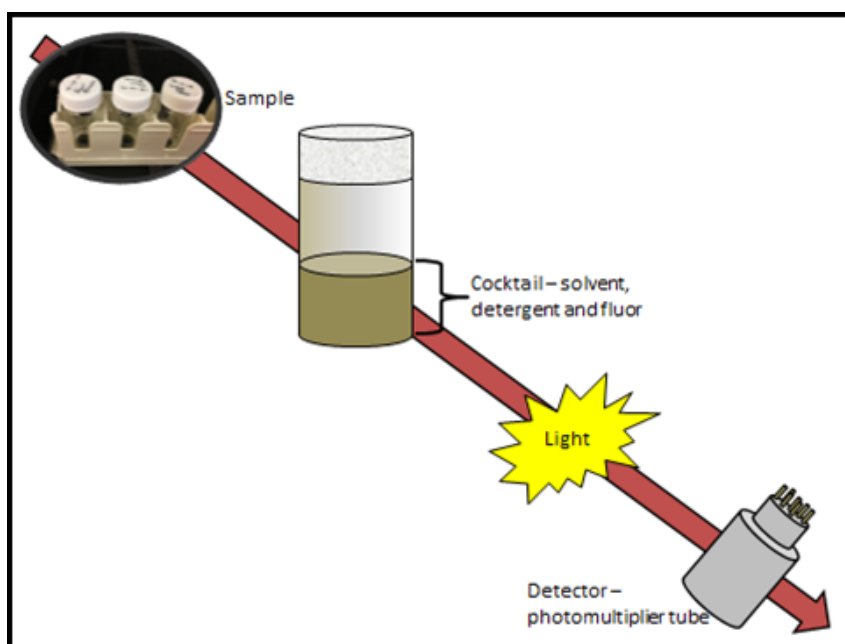


Figure 2.18. Mechanism behind liquid scintillation counting (LSC).

Method two was used for future experiments for reasons noted above. Subsequent to exposure, water in beakers was drained through a sieve (Fisherbrand, ISO 3310/1 250 μ M). Faeces and pseudo faeces were collected from sieve on a weighed section of tissue, and placed into pre-weighed tubes. Samples were freeze-dried (< 12 h, or until pressure is constant at 50-60 μ bar), re-weighed and rehydrated (1 mL, DI water). Mussels were separated into soft tissue (gill, mantle, adductor muscle, digestive gland and 'other' tissue), shell and internal mussel water (IMW). IMW refers to all water within the mantle cavity, fluid was collected by opening the shell, and draining fluid into a tube. Samples were re-weighed to get mL/individual. Soft tissue was dissected and placed into pre-weighed tubes, re-weighed and then homogenised in DI water (10 mL, this was noted as the most suitable volume to homogenise tissue in, Fig. 2.19). Shells were rinsed thoroughly, scrubbed using a sponge and placed into pre-weighed tubes and re-weighed, they were then crushed using a hammer and/or pestle and mortar. Shells were solubilised in concentrated nitric acid (5 mL, < 5 h) at room temperature with occasional shaking (200 rpm), and then diluted in DI water (15 mL).



Figure 2.19. Whole mussel tissue homogenised in different volumes of DI water (5, 10, 20 mL).

Soft tissue, shell, IMW or faeces solution (1 mL, in duplicate) was mixed with 4 mL scintillant (ScintLogic U, LabLogic Systems Ltd, UK) in scintillation vials (20 mL, Fisherbrand™ Borosilicate Glass), 4 mL was also added to water samples. Samples were left in dark for ~ 2 h prior to counting (Hidex 300SL), samples were read (10 s) in triplicate.

2.8.3.2 Liquid scintillation counting: Analysis of water and mussel tissue samples

To ensure accuracy of pipetting, a serial dilution of ^{32}P was carried out (see Fig. 2.20). Sample data from the Beckman Coulter was presented in DPM (disintegrations per min), and in counts per min (CPM) from the Hidex liquid scintillation counter. Activity concentrations were background corrected by blank subtracting from each sample, the blank was non-spiked fresh or seawater. Total activity (Bq) per whole tissue was calculated by dividing by 60 to acquire DPS (disintegrations per s), and then multiplying by the dilution factor where appropriate. All samples were decay corrected where applicable to determine total activity (Bq) per whole tissue (STA) on the day of sampling (tissue dissection following exposure) using the following formulae:

$$\text{STA} = \text{S0} \cdot 2^{((\text{N}/365)/\beta y)}$$

where S0 is the initial/starting sample activity (total activity [Bq] per whole tissue), N is the number of days to decay correct for (i.e. -4 is 4 d), and βy is the half-life of the radionuclide divided by 365 (days in one year, $^{32}\text{P} = 0.039$). Total activity (Bq) per gram of tissue (wet weight) was calculated by dividing the total activity (Bq) per whole tissue (decay adjusted) by the wet weight (g) of the tissue. In

accordance with Jaeschke and Bradshaw (2013), CPM values that fell below the blank were assigned an activity of 0.000.

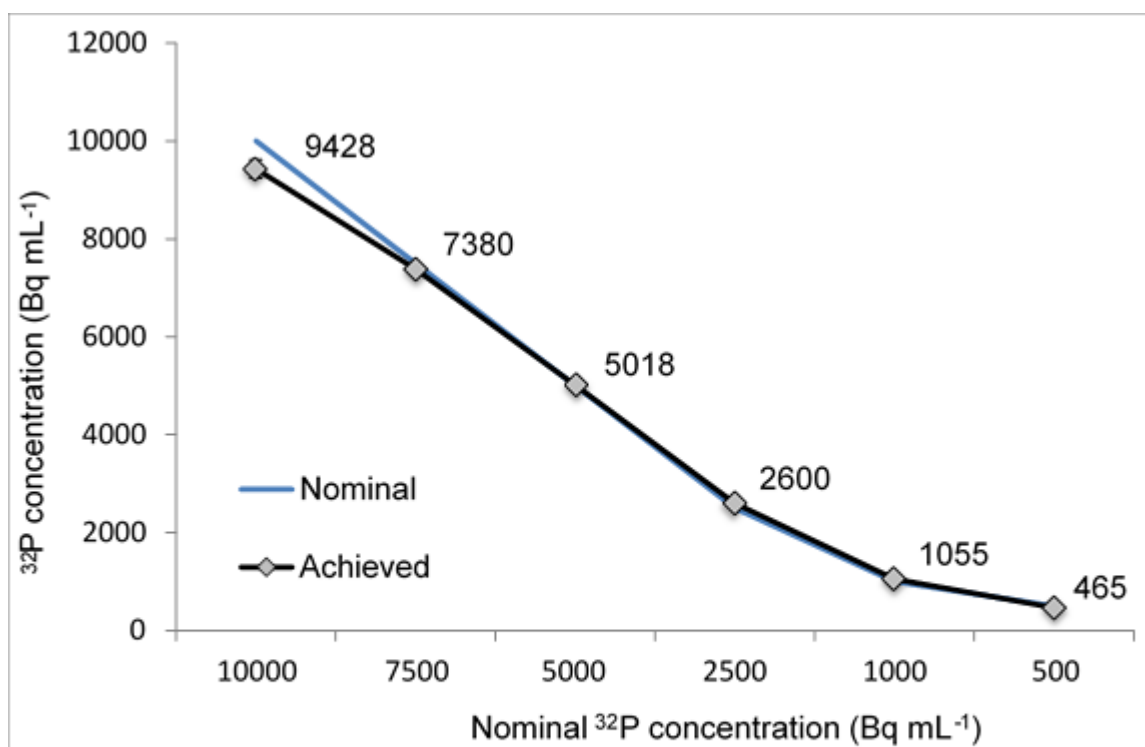


Figure 2.20. Series ^{32}P dilution showing nominal and achieved values. Error bars are SD of mean data.

2.8.4 Dosimetry calculations and the ERICA tool

The Environmental Risk from Ionising Contaminants: Assessment and Management (ERICA) Tool is a software system designed to assess the radiological risk to aquatic and terrestrial biota. This simplified dosimetry transfer model, which is organised into three tiers can be applied to extrapolate estimates of dose received by aquatic biota, from bioaccumulation data (Fig. 2.21).

The Tier 2 assessment module of the ERICA tool was used, ^{32}P was chosen as one of the ERICA tool's default isotopes. MG geometry parameters (Fig. 2.21) were adopted from Dallas et al (2016). Custom DP parameters (Fig. 2.21) were used for accurate dosimetry, such were mean measurements taken from

collected individuals from Bude, UK. Tissue specific geometry parameters (Table 2.4) were mean measurements taken during sampling. A radiation-weighting factor of 1 (ERICA tool's default for high-energy beta emitters) was used. The distribution co-efficient (K_d , sediment-water transfer) was set to 0 L kg^{-1} , as no sediment was present in the experimental design. Concentration ratio (CR) was set to 0 as actual tissue values were used, CR is defined as the activity concentration in biota (whole body, Bq kg^{-1}) divided by the activity concentration in water (Bq L^{-1}). Variable inputs required to calculate total dose rate per organism ($\mu\text{Gy h}^{-1}$) were activity concentration in water (Bq L^{-1}), activity concentration in sediment (Bq kg^{-1}), this is set to 0, and the activity concentration in organism (Bq kg^{-1}). For the latter, total activity (Bq) per beaker was divided by total mussel wet weight (g, including shell) per beaker, and then multiplied by 1000 to acquire Bq kg^{-1} .

Table 2.4. Table illustrating custom organism option in the ERICA tool; *D. polymorpha* digestive gland (DG) and *M. galloprovincialis* DG, occupancy factors and organism geometry. Ksib and Chi are scaling parameters, representing the lengths of the minor axes in terms of length of the major axis of the ellipsoid.

Parameter	Marine	Freshwater
	<i>M. galloprovincialis</i> DG	<i>D. polymorpha</i> DG
Mass (kg)	0.000176527	9.64833E-05
Height (m)	0.006	0.003
Width (m)	0.007	0.003
Length (m)	0.01	0.004
Occupancy		
Water-surface	0	0
Water	1	1
Sediment-surface	0	0
Sediment	0	0
Ksib	0.6	0.75
Chib	0.7	0.75

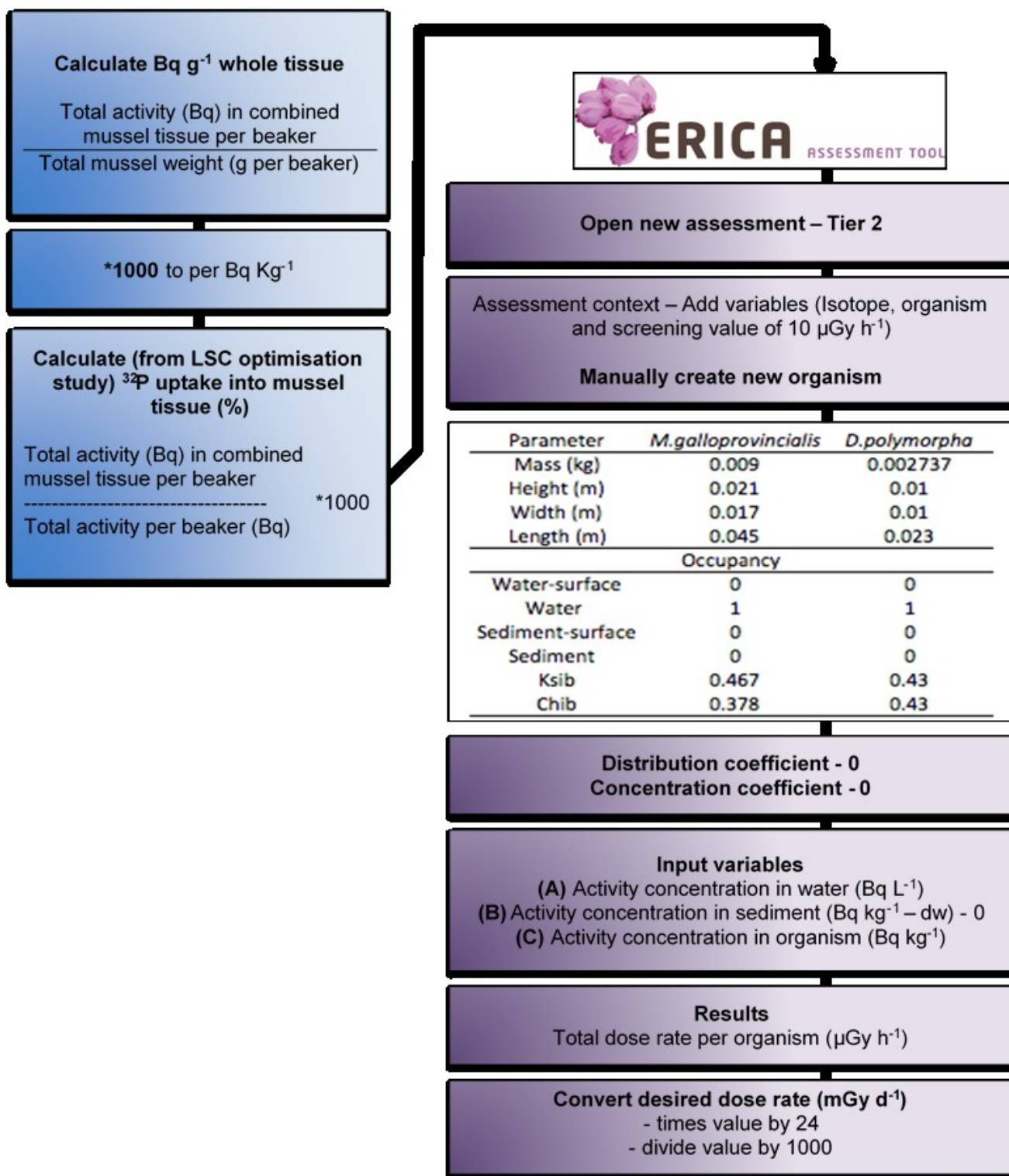


Figure 2.21. Diagram to show calculation of dose rates in *M. galloprovincialis* and *D. polymorpha*. Ksib and Chi are scaling parameters, representing the lengths of the minor axes in terms of length of the major axis of the ellipsoid.

2.8.5 Optimisation experiments

2.8.5.1 Pilot study

Starting activity levels (Bq L⁻¹) to give the desired dose rates of 0.10, 1, 10 mGy d⁻¹ were calculated using the ERICA tool (Fig. 2.21) using the criteria listed in Table 2.5 (Table 2.6). Activity levels were increased for the pilot experiment to 400 Bq/beaker (222 Bq L⁻¹), as to be able to determine low activity levels adequately using the Beckman Coulter. For accurate dosing and subsequent dosimetry calculations, there was an equivalent weight of individual soft tissue (wet weight, g) in each beaker. Before radioactive exposures, eleven individuals (per species) were dissected, soft tissue was blotted dry and weighed to four decimal places, shell was discarded. In accordance with Table 2.7, this equated to 3 MG (mean. wet weight 1.9 g) to 14 DP (mean. wet weight 0.41 g).

Table 2.5. ERICA tool criteria used for estimating dose rate from water concentrations. K_d is distribution coefficient, CR is concentration ratio.

	<i>M. galloprovincialis</i>	<i>D. polymorpha</i>
Tier	2	2
Isotope of interest	³² P	³² P
Environment	Marine	Freshwater
Organism	Mollusc – bivalve	Mollusc – bivalve
K_d (L kg ⁻¹)	21300	133
CR (1 Kg ⁻¹ , wet weight)	20000	60000
Occupancy	Water-surface 0 Water 1 Sediment-surface 0 Sediment 0	Water-surface 0 Water 1 Sediment-surface 0 Sediment 0
Radiation weighting factor	Defaults 1	Defaults 1

Table 2.6. Nominal dose rates (mGy d⁻¹), total dose (mGy 10 d⁻¹) and required water concentration (Bq L⁻¹) for ³²P exposures, using the ERICA tool default criteria.

Desired dose rate		Total dose (mGy 10d ⁻¹)	ERICA tool water concentration that gives the correct dose rate (Bq L ⁻¹)	
mGy d ⁻¹	μGy h ⁻¹ (for ERICA)		<i>M. galloprovincialis</i>	<i>D. polymorpha</i>
Control	n/a	n/a	n/a	n/a
0.1	4.16	1	0.578	0.183
1	41.60	10	5.780	1.825
10	416	100	57.800	18.250

Table 2.7. Weight of *M. galloprovincialis* and *D. polymorpha* (g).

<i>n</i>	Individual wet weights (g)	
	<i>M.galloprovincialis</i>	<i>D.polymorpha</i>
1	1.3038	0.5682
2	1.1049	0.3189
3	1.4683	0.2971
4	1.3489	0.3604
5	2.7929	0.567
6	1.8506	0.5991
7	2.51	0.2844
8	1.7813	0.2685
9	2.3642	0.2578
10	2.4871	0.6995
11	1.8458	0.2838
Average (g)	1.9	0.41
Individual/beakers	3	14
Soft tissue/beaker (g)	6	5.7

An 11-day pilot exposure was set up during September 2016 to determine bioaccumulation in mussel soft tissue over time, following exposure to ^{32}P . Mussels were maintained as in section 2.2. Following depuration mussels were transferred into 10 acid washed (5 per species), constantly aerated beakers, contained within appropriate shielding (2 L⁻¹ glass beakers, 1.6 MG or 7.8 DP L⁻¹).

Commercially available, radiolabelled-ATP (Adenosine triphosphate, γ - ^{32}P) was obtained from Perkin Elmer (PerkinElmer, UK) in batches of 9.25 MBq (specific activity: 370 MBq mL⁻¹) and used as the source of radioactive ^{32}P for our experimental purposes. Radiolabelled ATP was utilised in our experiments as (a) due to its readily bioavailable form would be accumulated readily into biological tissue, (b) the ATP itself would not cause biological damage as the radioisotope is almost chemically identical to the stable isotope, it therefore would not affect future experiments and (c) it would not affect the chemical composition (i.e. pH, salinity) of the sea/freshwater. Due to the short half-life of ^{32}P , stock solution was decay adjusted during the exposure.

Individuals were not fed during this exposure. Water quality parameters were pH 7.8 ± 0.32 , temperature 15.3 ± 0.7 °C, dissolved oxygen (DO) 95 ± 19.6 % and salinity 36.8 ± 0.28 for MG and pH 8 ± 0.29 , temperature 14.9 ± 0.5 °C, DO 98.5 ± 2.8 % and salinity 0.35 ± 0.08 for DP. On days 3, 5, 7, 9 and 11, soft tissue from individuals from two beakers, one per species was processed for LSC as in section 2.8.3.1 (Method 1. Water samples (100 μL , in duplicate) were taken each day.

Due to inefficient sensitivity of the liquid scintillation counter, combined with long tissue processing and counting times allowing for decay, ^{32}P was not evident in water samples. As displayed on Fig. 22, ^{32}P accumulation is significantly ($p <$

0.001) greater in MG soft tissue, than in DP over the duration of the experiment with an average total of 13.1 and 2.7 Bq g⁻¹, respectively.

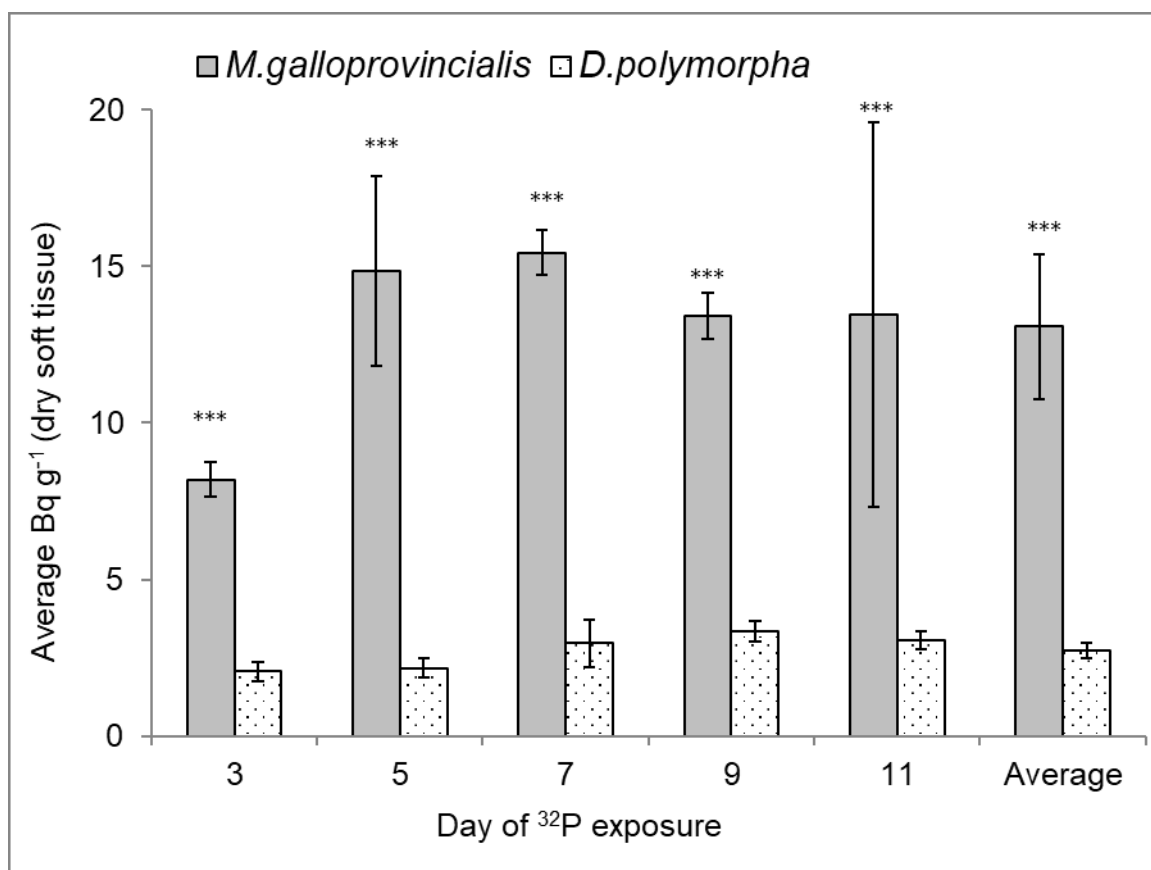


Figure 2.22. ³²P accumulation in *M. galloprovincialis* and *D. polymorpha* whole soft tissue, over time. Error bars show standard deviation of mean data. $n = 3$ (MG), 14 (DP). Significance (***) is $p < 0.001$ is shown between species on each sampling day.

³²P soft tissue concentrations remained relatively consistent over time, with no significant variation. This pilot study established firstly, and most importantly that ³²P could accumulate in marine and freshwater bivalve soft tissue to differing degrees. In terms of future experiments, several alterations to sampling procedures were required. Due to the short half-life of ³²P, time between the end of exposure and LSC of samples needed to be reduced. ³²P has a high counting efficiency (~ 98%), meaning a large percentage of beta particles emitted by

radioactive phosphorus are being counted via liquid scintillation (Curtis 1971). It is far less sensitive to the effects of quenching agents, whether chemical, physical or through differences in colour (Guinn 1957). It was therefore deemed unnecessary to solubilise and bleach samples prior to counting, as quenching will not affect the determination of activity levels. In addition, to adequately read the total activity within samples (tissue and water) spiked with low levels of ^{32}P , exposures would benefit from an increase in ^{32}P total activity (Bq L^{-1}).

As described in McDonald et al (1993) and Jha et al (2005), digestion and feeding can play a major role in nuclide uptake in biological systems. It is possible that this is the main ^{32}P accumulation pathway for bivalve species, as opposed to direct uptake from water. As noted by Smith et al (2011), it is important to acknowledge differing uptake pathways when determining contaminant accumulation in biological systems. Future experiments would benefit from the following alterations: (a) feeding study species and (b) use of a sensitive LSC able to count low radiation levels.

2.8.5.2 LSC optimisation study

In consideration of section 2.8.5.1, subsequent ^{32}P experiments utilised the Hidex 300SL LSC at the University of Portsmouth, UK or the University of Exeter Medical school, UK. A 6-day exposure ($n = 3$ [MG] and 14 [DP]) was set up during November 2016. Following acclimatisation, ^{32}P was added to produce a concentration of 2222 Bq L^{-1} (4000 Bq/beaker). Mussels were fed (days 3/5) during this exposure, as in section 2.2. Water changes (50 %) were carried out on day 3 and 5. Water samples (1 mL, in duplicate) were taken around 4 h (approx.) after each water change, and processed for LSC to determine water

activity concentrations. Water quality parameters were pH 7.9 ± 0.33 , temperature 15 ± 0.1 °C, dissolved oxygen (DO) 99.23 ± 1.53 % and salinity 36.6 ± 0.17 for MG and pH 8.3 ± 0.2 , temperature 14.8 ± 0.2 °C, DO 98.9 ± 2 % and salinity 0.4 ± 0.05 for DP. Samples (soft tissue, shell, IMW and water) were processed in accordance to section 2.8.3.1 (Method 2).

Average total activity in water was 1935 and 1437 Bq/beaker (1.8 L^{-1}), for MG and DP, respectively. This study suggests that 54% and 74% of ^{32}P is being accumulated into MG and DP individuals (Whole body, Fig. 2.23). From this we can employ the ERICA tool (section 2.8.4) to calculate correct water activity concentrations to give the expected dose rates of 0.10, 1 and 10 mGy d^{-1} for each species (Table 2.8).

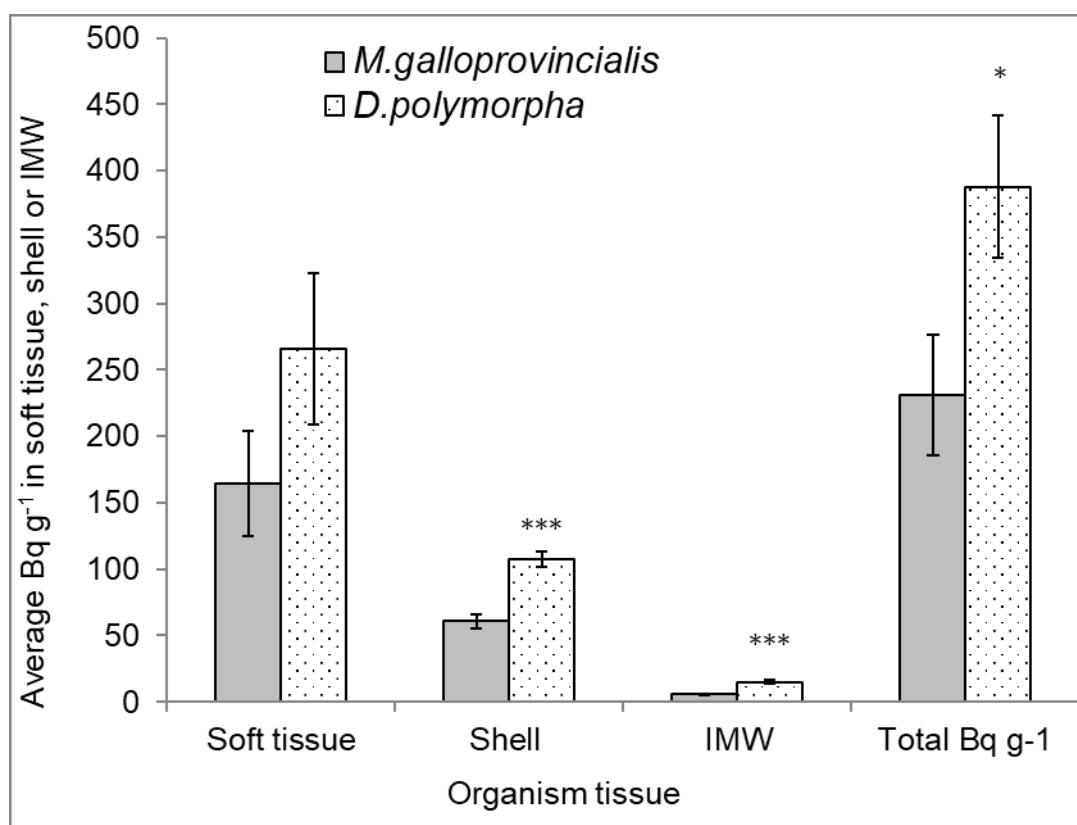


Figure 2.23. ^{32}P accumulation in *M. galloprovincialis* and *D. polymorpha* whole soft tissue, shell and internal mussel water. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) between species. Error bars show standard deviation of mean data. $n = 3$ (MG), 14 (DP).

Table 2.8. Water concentrations (Bq L⁻¹), calculated by the ERICA tool, that give to correct dose rates of 0.1, 1 and 10 mGy d⁻¹ in *M. galloprovincialis* and *D. polymorpha*.

	Nominal dose rate mGy d ⁻¹	ERICA tool water concentrations that give correct dose rate (Bq L ⁻¹)
<i>M. galloprovincialis</i>	0.1	709
	1	7090
	10	70900
<i>D. polymorpha</i>	0.1	571
	1	5710
	10	57100

2.9 Statistical analysis

All statistical analyses were carried out using the statistical software R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), <https://www.r-project.org/>), unless otherwise stated. All data was checked for normality distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with visual examination of QQ-plots. The non-parametric Kruskal-Wallis test was used if assumptions were not met; comparison between treatment groups was determined using a Dunn's pairwise comparison with Bonferroni correction. Where assumptions were met, a one-way ANOVA was run with Tukey's post hoc tests. Comparison between treatment groups was determined using a Wilcoxon rank sum test with Holm-Bonferroni correction. Regression analysis using a Pearson correlation coefficient was used to determine any correlations between variables, all figures displaying correlations include a best fit line (regression line). Level of significance for all tests was set at $p < 0.05$ (*) and data presented as mean \pm standard deviation, unless otherwise stated.

Chapter 3

Assessing relative sensitivity of marine and freshwater bivalves following exposure to copper: Application of classical and novel genotoxicological biomarkers



Under review

Vernon, EL and Jha, AN. 2018. Assessing relative sensitivity of marine and freshwater bivalves following exposure to copper: Application of classical and novel genotoxicological biomarkers. Mutation research/Genetic Toxicology and Environmental Mutagenesis.

3.1 Introduction

Identification of most sensitive natural species following exposure to anthropogenic contaminants is important from an environmental protection point of view (Jha, 2004, 8; Canty et al. 2009; Dallas et al. 2013). In recent years, there has been growing regulatory and scientific concerns for those contaminants which have the potential to exert carcinogenic, mutagenic and endocrine disrupting effects (Dallas et al. 2013). In this context, it is also emerging that the contaminants could simultaneously induce their toxicity in a variety of ways, i.e. the same contaminant could induce carcinogenic, mutagenic, immunotoxic and endocrine disrupting effects (Jha 2008).

Metals are an important group of ubiquitous contaminants to which biota are exposed in different habitats and ecological niches (Bolognesi et al. 1999; Dallas et al. 2013). Exposure to metals can induce a variety of detrimental biological effects via a range of mechanisms, including through the generation of ROS. In addition to damage to cellular components such as lipids and proteins, DNA damage (either directly or through generation of ROS), inhibition of DNA repair capacity and disruption of cell cycle control are of particular concern (Stohs and Bagchi 1995; Azqueta et al. 2009; Dallas et al. 2013; Xu et al. 2016). Although various metals are essential for biological processes, copper (Cu) in particular being important for growth, metabolism and enzymatic activities (Cid et al. 1995; Gaetke and Chow 2003; Bopp et al. 2008; Kim et al. 2008), can be highly toxic to organisms including bivalves at higher concentrations (Bolognesi et al. 1999; Al Subiai et al. 2011). Cu (Cu^{2+}) has been the focus of extensive research as a widespread contaminant, present in coastal and inland water bodies globally and is considered to be of greater environmental concern compared to other contaminants such as pharmaceuticals (Eisler 1997; Donnachie et al. 2016).

Total dissolved Cu in contaminated environments have been found to reach concentrations of 689 $\mu\text{g L}^{-1}$ (Bryan and Gibbs 1983), with permitted levels in England and Wales ranging from 1–28 $\mu\text{g L}^{-1}$ in freshwater (dependant on water hardness), and 5 $\mu\text{g L}^{-1}$ in seawater (DEFRA 2014).

The link between metal (in particular Cu) exposure and genotoxicity, as determined by induction of MN and DNA strand breaks (as determined by comet assay) has been well documented in a range of aquatic species, including bivalves (Geret et al. 2002; Brown et al. 2004; Villela et al. 2006; Al-Subiai et al. 2011; Trevisan et al. 2011; Mai et al. 2012; Vosloo et al. 2012; Maria et al. 2013; Brooks et al. 2015; Lewis et al. 2016). Induction of DNA strand breaks has been noted at environmentally relevant Cu concentrations in several bivalve species, including *Mytilus* spp., (18-56 $\mu\text{g L}^{-1}$), *Perna perna* (37.5-50 $\mu\text{g L}^{-1}$), *Limnoperna fortunei* (375-750 $\mu\text{g L}^{-1}$) and *Crassostrea gigas* (embryos, 1-20 $\mu\text{g L}^{-1}$) (Bolognesi et al. 1999; Villela et al. 2006; Al-Subiai et al. 2011; Trevisan et al. 2011; Vosloo et al. 2012; Sussarellu et al. 2018).

Species-specific differences in bioaccumulation and resultant biological response following exposure to environmental contaminants have been reported for different marine bivalves (Pellerin and Amiard 2009; Brooks et al. 2015; Marisa et al. 2018). Despite the fact that (a) freshwater and marine bivalves are comparable in their external and internal anatomical structures (Vernon et al. 2018) and (b) contaminants including metals are highly relevant to both marine and freshwater environments, there have been no attempts to compare the relative bioaccumulation and biological responses in these ecologically relevant organisms. As noted in section 1.4.1, the marine, *Mytilus galloprovincialis* (MG) and freshwater mussel, *Dreissena polymorpha* (DP) were chosen for this study as ecologically relevant representatives of both coastal and inland water bodies

(McDonald et al. 1991; Binelli et al. 2015). As sessile, filter feeding organisms, both species are used extensively in monitoring programmes and in toxicological research as accurate bioindicators of their environmental surroundings (NOAA 2012). The physiology, anatomy and ecology of MG and DP is well understood and their effectiveness within ecotoxicological studies well documented (Binelli et al. 2015), nevertheless, the relative sensitivity of marine and freshwater bivalves, in terms of genotoxic damage is yet to be fully explored. As suggested by various authors the use of two (or more) species should be considered as more robust, realistic approach for ecotoxicological studies (Chapman 2002; Solomon and Sibley 2002; Schnug et al. 2014).

The extent of damage in individuals evident at higher levels of biological organisation, subsequent to DNA strand breaks, is dependent on a range of factors including exposure period, contaminant concentration, rate of uptake, metabolism, accumulation and the efficiency of repair mechanisms (Jha 2008). In recent years, immunostaining techniques such as γ -H2AX and 53BP1 have been utilised as sensitive markers of DNA DSBs (Gerić et al. 2014; Oommen et al. 2016b). The genotoxicity of various contaminants (e.g. metals, nanoparticles, insecticides and radionuclides) using induction of γ -H2AX as a biomarker has been displayed in zebrafish (*Danio rerio*) ZF4 cells, adult zebrafish liver tissue and retina, and fathead minnow (*Pimephales promelas*) juveniles (Choi et al. 2010; Pereira et al. 2012, 13; Gagnaire et al. 2017; Paravani et al. 2018). Concentration-dependent DNA damage in ZF4 cells was evident following aluminium (10-100 μ M) and cadmium (1- 100 μ M) exposures (Pereira et al. 2013). γ -H2AX foci induction followed a similar trend, where number of foci per cell increased with aluminium or cadmium concentration up to 30 μ M, and then decreased in 50-100 μ M treatments (Pereira et al. 2013). Such findings aid to our

limited knowledge of DSB-repair mechanisms, subsequent to exposure to environmentally relevant contaminants in non-mammalian models. In spite of usefulness of this assay, particularly when validated alongside classical genotoxicity assays (i.e. comet or MN assays), this highly sensitive approach is yet to be applied in aquatic invertebrates.

In light of the above information, following exposure to a range of Cu^{2+} concentrations (referred to as Cu throughout text) the objectives of this study were (a) to investigate the relative tissue specific accumulation of Cu in both bivalve species, (b) to establish a concentration-response curve for genotoxic responses in the adult life stages of the species (c) to determine relative sensitivity between the two species for genotoxic responses using a range of genotoxicity parameters (i.e. induction of MN, Comet and $\gamma\text{-H2AX}$) (d) to correlate the nominal Cu concentrations in water with bioaccumulation and observed genotoxic responses and (e) to determine potential correlations between different genotoxicity parameters studied. With regards to species variation, we hypothesised firstly that little disparity in genotoxic response will be evident. Secondly, with increased DNA damage (DNA strand breaks and MN formation), a greater induction of $\gamma\text{-H2AX}$ foci will be evident.

3.2 Materials and methods

3.2.1 Chemicals and suppliers

All chemicals and reagents were purchased from Fisher Scientific UK, Anachem Ltd. UK, Sigma-Aldrich Ltd. UK, VWR International Ltd USA or Greiner Bio-One Ltd UK, unless stated otherwise. Product details are mentioned in text as appropriate.

3.2.2 Mussel exposure conditions

Four 10-day Cu exposure experiments were performed between April 2016 and December 2017, the first two to determine genotoxic responses and the latter for tissue specific Cu accumulation measurements. Adult MG (shell length 44.5 ± 6.5 mm) and DP (shell length 26.7 ± 4.31 mm) were collected from Trebarwith strand (as in Dallas et al (2013)) and Bude, Cornwall, UK (Chapter 2, section 2.2), respectively (Vernon et al. 2018). DP and MG were maintained in accordance to chapter 2, section 2.2.

Exposures of bivalves to Cu were staggered by one week for ease of analysis and logistical reasons. Subsequent to a two-week depuration period after collection, individual mussels were transferred into twelve acid washed glass beakers, in triplicate (i.e. 1.7 mussels L⁻¹), containing 1.8 L⁻¹ of water and aerated (Dallas et al. 2013). Individuals were then acclimatised for 48 h prior to exposure. Beakers were labelled and assigned to one of the four treatment groups: 0 (control), 18, 32 and 56 µg L⁻¹ Cu (as CuSO₄.5H₂O, 99% purity), there were three replicates per treatment (Fig. 3.1). Cu concentrations were selected in accordance to previous work from our laboratory and in line with environmental realistic values (Al-Subiai et al. 2011).

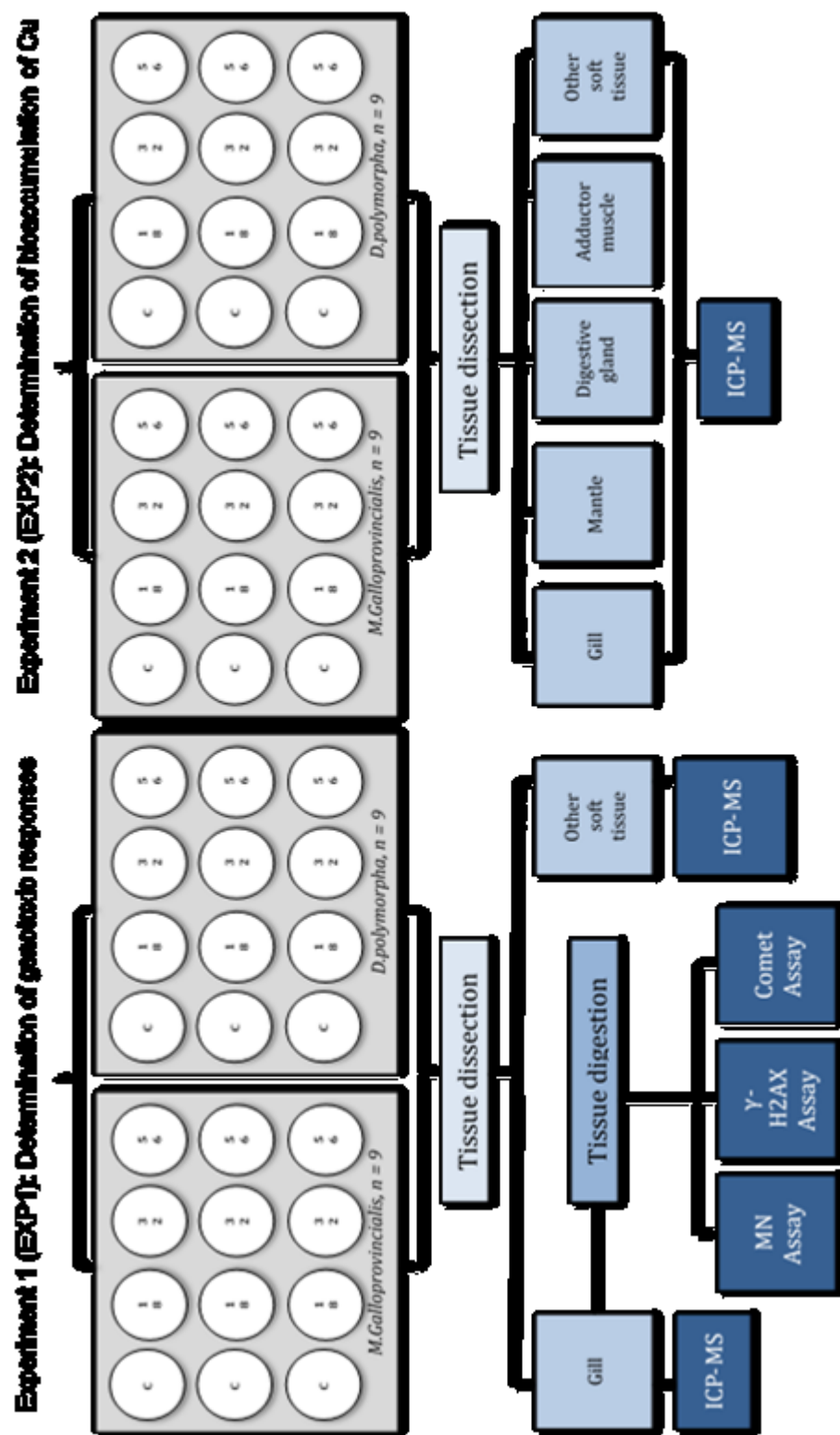


Figure 3.1. Overall experimental design: Experiment 1 (EXP1): Determination of Genotoxic responses; Experiment 2 (EXP2): Determination of bioaccumulation of copper (Cu).

Water changes were performed every alternate day with the appropriate Cu added to meet desired concentration. Water samples were taken 1 h after each water change, and processed for determination of Cu concentrations as described in section 2.7.2. Water quality parameters were measured routinely, before and after water changes, as outlined on table 3.1.

Table 3.1. Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]) and copper concentrations in water ($\mu\text{g L}^{-1}$). Data are presented as mean \pm standard deviation (SD).

Water parameters	EXP 1: Genotoxicity		EXP 2: Bioaccumulation	
	<i>M.galloprovincialis</i>	<i>D.polymorpha</i>	<i>M.galloprovincialis</i>	<i>D.polymorpha</i>
pH	8 \pm 0.11	8 \pm 0.17	8.1 \pm 0.06	8.1 \pm 0.06
Temp ($^{\circ}\text{C}$)	15.4 \pm 1.22	14.8 \pm 0.28	15.0 \pm 0.24	14.8 \pm 0.32
Salinity	33.2 \pm 0.32	0.3 \pm 0.01	33.1 \pm 0.07	0.3 \pm 0.01
DO (%)	96.5 \pm 2.47	98.8 \pm 0.57	99.0 \pm 0.64	99.0 \pm 0.72
Copper water conc. ($\mu\text{g L}^{-1}$)				
Control	0.1 \pm 0	3.1 \pm 0.24	7.3 \pm 0.38	3.6 \pm 0.43
18 ($\mu\text{g L}^{-1}$)	12.5 \pm 1.49	14.8 \pm 2.82	20.2 \pm 0.74	19.1 \pm 2.9
32 ($\mu\text{g L}^{-1}$)	29 \pm 2.87	25.2 \pm 1.82	30 \pm 1.03	31 \pm 5.41
56 ($\mu\text{g L}^{-1}$)	51.6 \pm 6.23	49.8 \pm 3.96	47.5 \pm 2	51.8 \pm 10.06

3.2.3 Sampling procedures

To determine genotoxicity (EXP1) gill tissue was extracted and stored as followed until use: $\frac{1}{2}$ gill stored on ice for digestion, $\frac{1}{2}$ gill stored in pre-weighed tube, placed in 60 $^{\circ}\text{C}$ incubator for subsequent ICP-MS determination of Cu content (section 2.7.1). For Cu accumulation (EXP2), tissue was dissected into gill, mantle, digestive gland, adductor muscle and other soft tissue, and processed for ICP-MS as described in section 2.7.1.

3.2.4 Biological assays

3.2.4.1 Isolation of gill cells for genotoxicity assays

The procedure to obtain gill cells for genotoxic assays was adopted from previous studies (Vincent-Hubert et al. 2011), and outlined in section 2.3.2. Supernatant was then used in subsequent assays providing cell viability, checked using the Trypan Blue exclusion dye assay (section 2.4) (Strober 2001) was <90% across all treatments (data not shown).

3.2.4.2 Comet assay to determine DNA strand breaks

The comet assay was performed using gill cell suspension (150 μL), as described in section 2.5.2.

3.2.4.3 Analysis of micronuclei (MN) induction

Gill cell suspension was adhered and fixed as described in section 2.5.1, before staining with 20 μL ethidium bromide (20 μL of 20 mg L^{-1}). Cells ($n = 500$) were scored per slide and results are reported as mean MN per 1000 cells, in keeping with other data from our research group (Dallas et al. 2013).

3.2.4.4 Induction of γ -H2AX foci

γ -H2AX foci were determined in gill cells, following procedures outlined in 2.5.3. All slides, including procedural blanks were coded and scored at random and 50 cells were counted per individual/slide.

3.2.5 Determination of Cu concentration in soft tissues and in water samples

3.2.5.1 Cu analysis in tissues using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Cu determination in tissue samples (i.e. gill, mantle, digestive gland, adductor muscle and 'other' soft tissue) and water was carried out using ICP-MS as described before in publications from our laboratory (Al-Subiai et al. 2011; Dallas et al. 2013; D'Agata et al. 2014) and in section 2.7. Procedural blanks, along with a certified reference material (TORT-2, lobster hepatopancreas) were run alongside samples.

3.2.5.2 Determination of Cu in water samples using ICP-MS

Cu samples in water were determined following procedures outlined in section 2.7.2.

3.2.6 Statistical analysis

All statistical analyses were carried out using the statistical software R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), <https://www.r-project.org/>), in accordance to principles detailed in section 2.9. Briefly, data was checked for normality distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test). Appropriate tests were used to determine comparison between treatment groups and correlations between variables.

3.3 Results

No spawning of mussels occurred during the duration of the experiments and mortality remained low throughout, with one fatality in the highest Cu treatment (MG, 56 $\mu\text{g L}^{-1}$, EXP1). Metal concentration and water quality measurements are displayed in table 3.1, results of the ICP-MS analysis confirmed that achieved values were in line with expected Cu concentrations across all treatments.

3.3.1 Tissue specific Cu accumulation

After a 10-day exposure, a substantial accumulation of Cu in both bivalve species was observed. Fig 3.2 highlights the variable nature of tissue specific uptake. Cu accumulation occurred in a concentration dependant manner in all tissues but the mantle of both species, MG 'other' soft tissue, and DP adductor muscle. Cu uptake varied between tissue, and between species. Concentrations ranged between 8.7 and 311.4 $\mu\text{g g}^{-1}$ (dry weight, d.w), with the highest levels evident in gill and digestive gland, independent of species. In the highest treatment group (56 $\mu\text{g L}^{-1}$), accumulation varied in the order of gill > digestive gland > other soft tissue > mantle > adductor muscle in MG, and digestive gland > gill > mantle > other soft tissue > adductor muscle in DP. In terms of whole soft tissue (d.w, sum of all tissue), DP had a greater degree of accumulation than MG in all but the highest treatment group. In the 32 $\mu\text{g L}^{-1}$ treatment, the accumulation of Cu in DP (458 $\mu\text{g g}^{-1}$) was 1.2 times higher than in MG (382 $\mu\text{g g}^{-1}$), and approx. 2 times higher in control treatments.

Biological response clearly correlated with the level of Cu accumulation in gill tissue, where the highest genotoxic response was found in mussels exposed to the two highest Cu concentrations (Fig. 3.3).

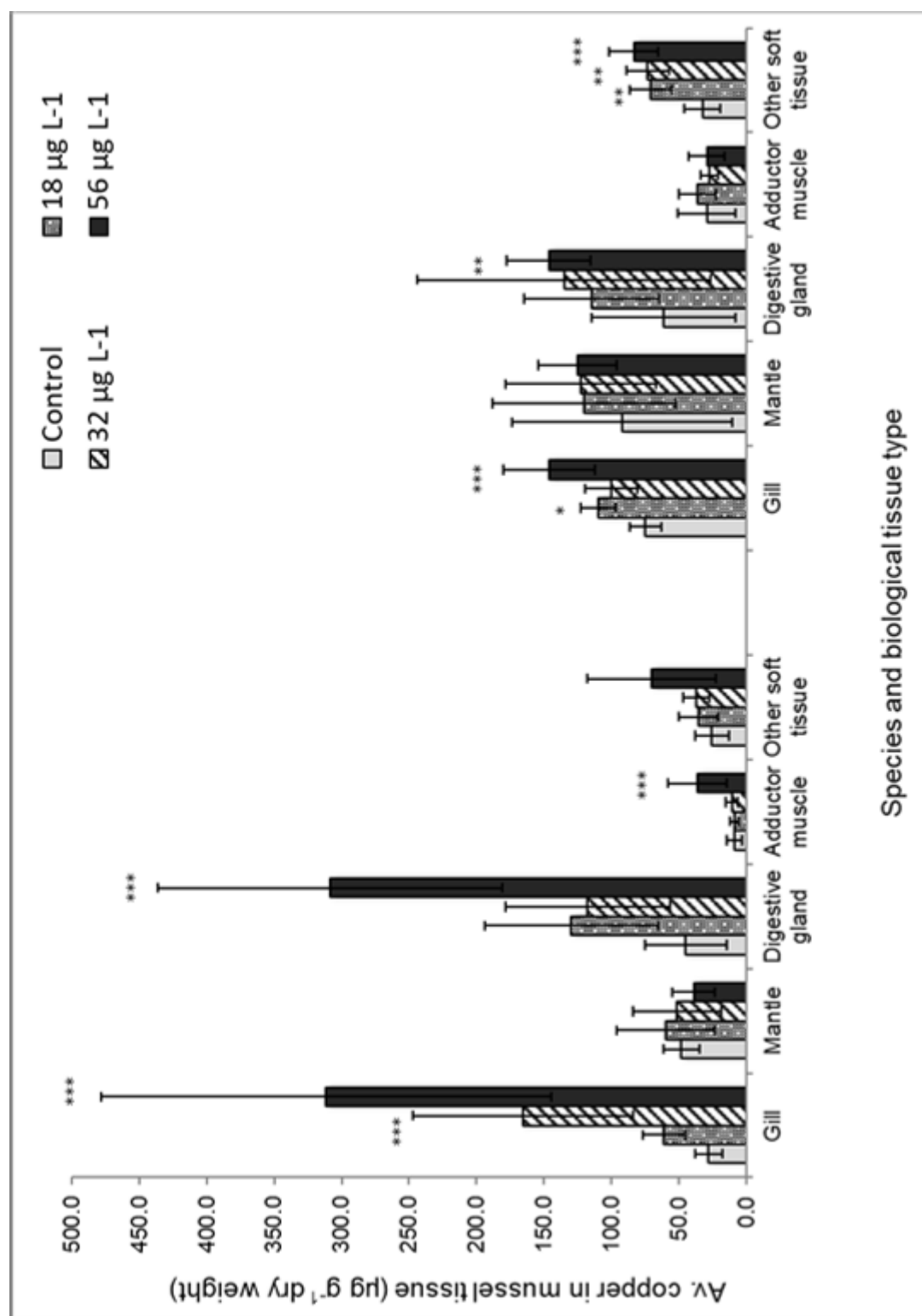


Figure 3.2. Tissue specific accumulation of copper in *M. galloprovincialis* (left) and *D. polymorpha* (right), microgram per gram of mussel tissue (dry weight) in control and exposed treatment groups. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data. $n=9$.

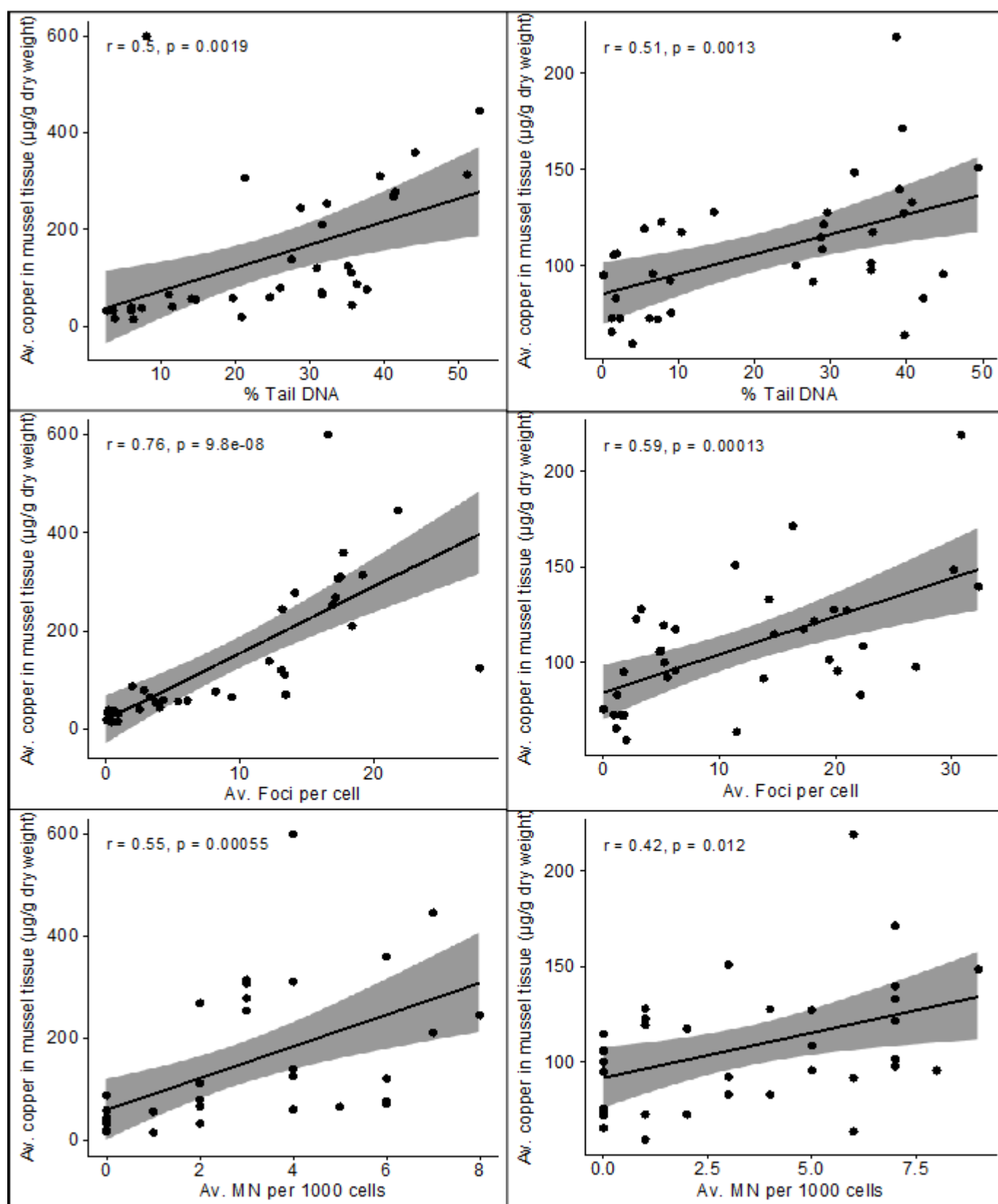


Figure 3.3. Pearson's correlation analyses of Cu accumulation in gill tissue. Top to bottom: DNA damage (% tail DNA), induction of γ -H2AX foci and micronuclei (MN) formation in *M. galloprovincialis* (left) and *D. polymorpha* (right). $n = 9$.

3.3.2 Genotoxic effects and repair capacity in mussel gill cells

Fig. 3.4A, B and C show the mean (\pm S.D) % tail DNA damage, micronuclei per 1000 cells and γ -H2AX induction respectively in the gill cells of the two species following exposure to varying Cu concentrations for 10 days. Both the species showed genotoxic effects following Cu exposure compared to controls. Independent of species, a concentration dependent increase was evident across all genotoxic biomarkers in response to Cu ($p < 0.001$).

Although a genotoxic response was evident in the lowest Cu treatment for all biomarkers studied, a significant response was evident only for 32 and 56 $\mu\text{g L}^{-1}$ treatments compared to the controls. Interestingly, there was no significant difference in response between the 32 and 56 $\mu\text{g L}^{-1}$ treatments in either species. In the highest concentration (i.e. 56 $\mu\text{g L}^{-1}$ Cu) the average level of induced γ -H2AX foci per cell was 18 ± 6 and 22 ± 8 foci per cell in MG and DP, compared with 0.4 ± 0.3 and 1 ± 0.6 foci per cell for control treatments. For both species, the observed response for γ -H2AX showed a strong correlation with DNA damage ($p < 0.001$) and MN formation ($p < 0.001$, Fig. 3.5). The % tail DNA in the highest treatment averaged around 37% (both species), as expected a low degree of damage was evident in control treatments. DNA damage in individuals exposed to the highest Cu concentrations was 5 and 9.5 times higher in MG and DP, in comparison to the control. In terms of species comparison, despite disparity in Cu accumulation among the tissues, there was little variation in genotoxic response in the cells. The only significant variation occurred between γ -H2AX foci induction in control cells ($p < 0.05$).

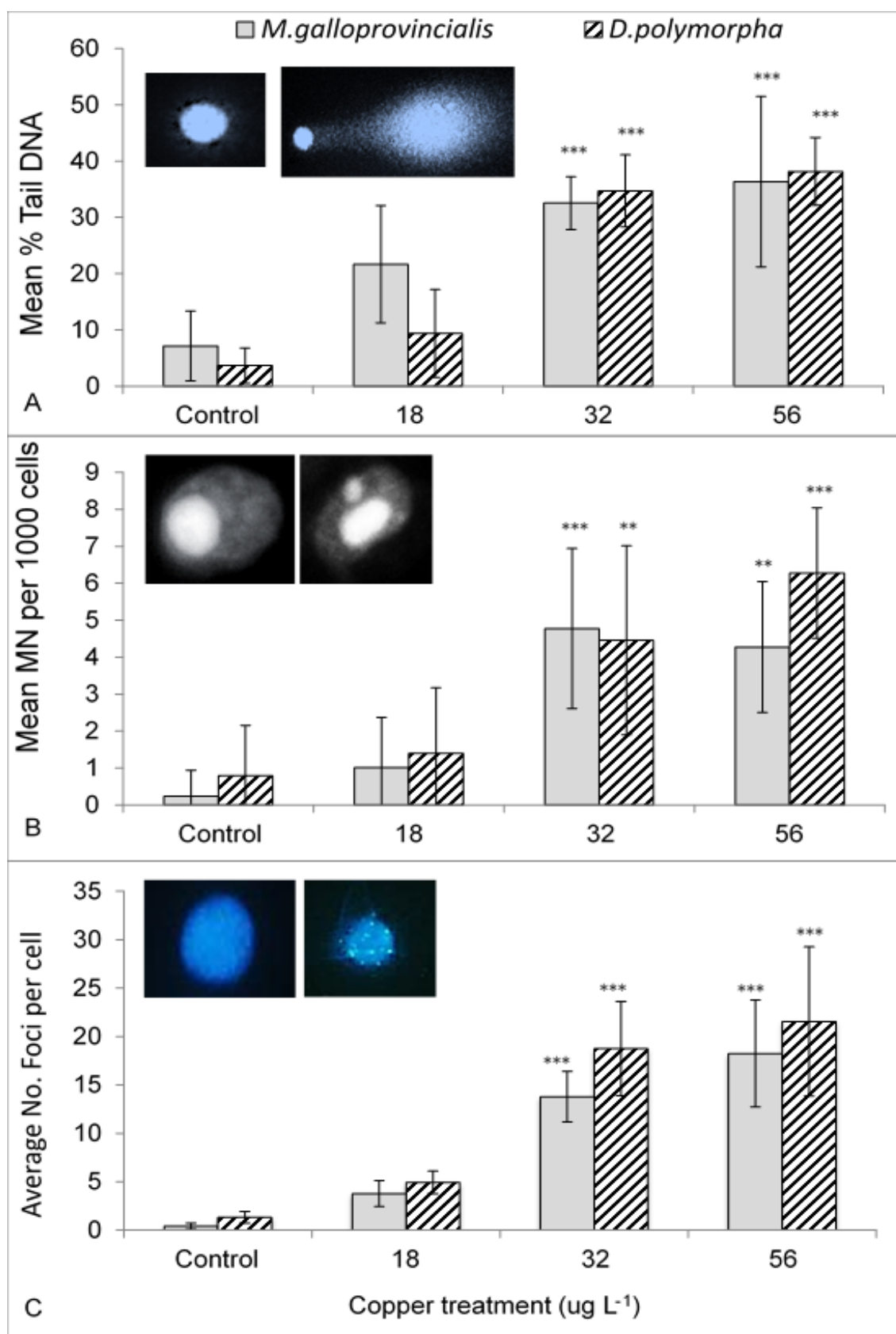


Figure 3.4. Genotoxic responses in *M. galloprovincialis* and *D. polymorpha* gill cells following a 10 day exposure to copper (Cu). (A) DNA damage (% tail DNA), (B) Induction of micronuclei (MN) and (C) induction of γ -H2AX foci Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data. Images show (left) control cell and (right) damaged cell. $n = 9$.

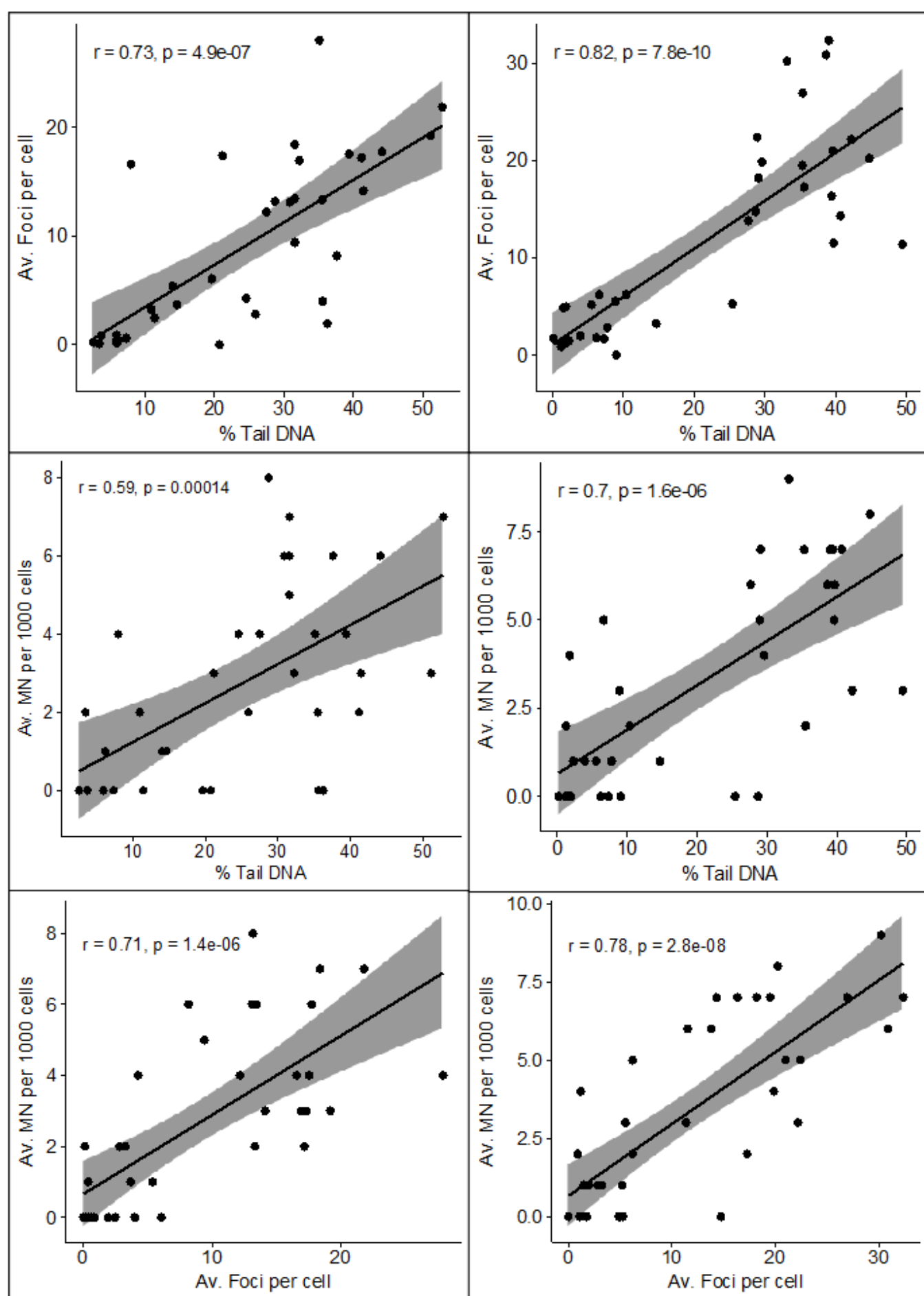


Figure 3.5. Pearson's correlation analyses. Top to bottom: % tail DNA and induction of γ -H2AX foci; % tail DNA and induction of MN and induction of MN and γ -H2AX in *M. galloprovincialis* (left) and *D. polymorpha* (right). $n = 9$.

3.4 Discussion

3.4.1 Tissue specific Cu accumulation

It is well accepted that following uptake, waterborne contaminants are not uniformly distributed among the tissues due to their inherent metabolic capabilities (Jha et al. 2006; Faggio et al. 2018). The presence of metals in the sediments has also been correlated with their accumulation in soft tissues of bivalves under field conditions (Dallas et al. 2013). As expected, in our study Cu concentrations varied among the tissues (Jing et al. 2006; Martins et al. 2011; Di Salvatore et al. 2013; Jorge et al. 2016). The highest Cu concentrations were found in the gill and digestive gland, particularly in the MG 56 $\mu\text{g L}^{-1}$ treatment. In this treatment, 40% (MG) and 28% (DP) of accumulated Cu was located in the digestive gland, this is in contrast to previous reports where Cu in DP had been shown to predominately accumulate in the foot (included in other soft tissue in this study), followed by gill and digestive organs (Gundacker 1999). Mussel digestive systems are well known to harbour contaminants via dietary uptake pathways (Viarengo et al. 1981; Regoli 1998; Marigomez et al. 2002; Faggio et al. 2018). Apart from inherent metabolic differences, uptake and bioaccumulation is dependent on many factors including bioavailability, uptake mechanism and biological factors (e.g. weight, gender, reproductive stage, feeding habits etc.). These factors may help explain disparity between the studies (Jha 2008).

In line with findings from Sanders et al (1994), Zorita et al (2007) and Al-Subiai et al (2011) a high degree of Cu bioaccumulation was evident in gill tissue of both species. Bivalve gills due to proximity to surrounding media, and therefore the primary sites of uptake of dissolved Cu are often regarded as a key tissue of interest in ecotoxicological studies. In contrast to Al-Subiai et al (2011), who found reduced Cu accumulation in 56 mg L^{-1} compared to 32 mg L^{-1} treatment

across all tissue (adductor muscle, digestive gland, gills), in our study accumulation increased in a concentration dependant manner in accordance to external Cu concentration (all but the MG mantle and DP adductor muscle tissue, Fig. 3.2). These differences may result from varying experimental procedures, including shorter exposure length or varying feeding regimes (i.e. individuals fed/not fed). Cu bioaccumulation and subsequent biological response in *Mytilus* spp. has been investigated by Brooks et al (2015), who found an increased rate of bioaccumulation in *M. trossulus* compared to *M. edulis/galloprovincialis* (4 d, 500 $\mu\text{g L}^{-1}$), and a higher prevalence of MN in *M. edulis* compared to *M. trossulus*. The mussels included in this study were collected from three different geographical locations (i.e. Norway and the Basque country, Spain). Hybridization and introgression of geographically dispersed species could play an important role in the bioaccumulative potential of contaminants and may explain the differences found between biological studies (Al-Subiai et al. 2011; Brooks et al. 2015; Larsson et al. 2018). A more complete introgression analysis that is not assessed by the Glú-5 gene (nuclear DNA marker used to characterise *Mytilus* spp.) may help to identify species differences that affect contaminant uptake (Kijewski et al. 2009, 2011).

Whole soft tissue Cu concentrations were reflective of exposure, with accumulation occurring in a concentration dependant manner. Interestingly, in all but the highest treatment, DP showed a greater degree of Cu accumulation in whole soft tissue, uptake in DP also appeared to be more evenly distributed across specific tissues. It is important to note that differential Cu speciation in water bodies may affect its bioavailability and subsequent toxicity to aquatic biota. The physical and chemical form of Cu, with focus on the bioavailable ionic form (Cu^{2+}), varies between salt and freshwater environments, becoming more

abundant at lower salinities (Grosell et al. 2007). The influence of water parameters (i.e. pH, salinity, dissolved organic carbon (DOC), alkalinity) in affecting Cu bioavailability and toxicity may explain disparities present within our data, along with differential physiology (Welsh et al. 1993; Erickson et al. 1996; Santore et al. 2001). As certain parameters (e.g. DOC) were not determined during this experiment, we are unable to determine if the species disparity resulted from varying water chemistry, especially DOC or differential species sensitivity. In both species independently, however correlation between accumulations of Cu in soft tissues with increasing genotoxicity in gill cells were evident.

3.4.2 Cu induced genotoxicity in gill cells

The capacity of Cu to induce chromosomal damage in a range of cell types has previously been reported in MG (Brooks et al. 2015; Ruiz et al. 2015), and in DP in response to a range of contaminants (Mersch et al. 1996; Mersch and Beauvais 1997; Bolognesi et al. 2004). As mentioned earlier, Cu accumulation in mussel tissue is significantly correlated with the adverse genotoxicological effects noted in both the species (Fig. 3.3). Cu toxicity in marine bivalves has been demonstrated extensively in scientific literature (Brown et al. 2004; Al-Subiai et al. 2011; Brooks et al. 2015; Digilio et al. 2016), along with freshwater species (Clayton et al. 2000; Bouskill et al. 2006; Sabatini et al. 2011). In line with previous studies, significant effects (i.e. DNA strand breaks and MN induction) were evident in both marine and freshwater mussels exposed to the highest Cu concentrations (32 and 56 $\mu\text{g L}^{-1}$), with a 4-9 fold increase in DNA damage relative to controls. The genotoxicity of Cu in mussel gill cells may be related to the overproduction of ROS, leading to oxidative damage in the form of SSBs and

DSBs, base modifications or oxidation of bases (Lloyd and Phillips 1999). Furthermore, Cu^{2+} is known to bind to DNA, forming adducts (Sagripanti et al. 1991).

While MG individuals showed greater Cu concentrations in gill tissue, there was a high degree of comparability between species response suggesting that Cu toxicity is not necessarily related to accumulation. In terms of the comet assay results, both species showed around 34-38% damage (% Tail DNA) in two highest Cu concentrations. Background levels of DNA damage in the controls were around 7 ± 6 (MG) and 4 ± 3 % (DP, % Tail DNA), relatable to previous findings (Klobučar et al. 2003; Jha et al. 2006; Dallas et al. 2013), this suggests general good health of the unexposed (control) individuals. Interestingly, Cu genotoxicity was not significantly evident at the lowest concentrations ($18 \mu\text{g L}^{-1}$), in either species. This is contrast to Anjos et al (2014), who found significantly increased DNA damage in sea anemone (*B. cangicum*) pedal disk cells exposed to much lower Cu concentrations ($7.8 \mu\text{g L}^{-1}$, 24 h). Our data was also in contrast to that of Al-Subiai et al (2011), who noted significantly increased DNA damage in *Mytilus edulis* at $18 \mu\text{g L}^{-1}$, following a 5 day Cu exposure. As mentioned above, several biological and physico-chemical factors could account for these differences (Jha 2008).

As previously mentioned, direct species comparison is limited due to differing water chemistry, potentially altering bioavailability. In addition, tissue Cu concentration is not necessarily a reliable indicator of toxicity. It is only a proportion of metal that interacts with sensitive target molecules (i.e. DNA) that induces a toxic effect. Despite this, it is clear that even at low tissue concentrations a genotoxic response is present in both species. Larsson et al (2018) found a lack of difference in response to environmental stressors between

marine mussels collected from reference and contaminated sites (i.e. sewage treatment plants, harbours) around the Baltic Sea region. The authors suggest that the presence of strong introgression between the two *Mytilus* taxa, along with adaptation to the specific environmental conditions could have accounted for this lack of differential sensitivity (Larsson et al. 2018). In our study, the similarity in DNA damaging effects suggests a similar mechanism of action in response to pollutants in the two species.

This study highlights the potential of zebra mussels (DP) as a freshwater equivalent to *Mytilus* species. Due to their ubiquitous, invasive nature they are regarded as a fairly tolerant species (Clayton et al. 2000). As with *Mytilus* spp., a range of cell types from gill to haemocytes can be successfully utilised in biological assays. As expected, in this study damage to DNA and MN formation was significantly correlated, in both species. Previous studies from our laboratory have reported significant correlations between induction of MN and DNA strand breaks in mussels and sea stars following exposures to environmentally relevant metals and pharmaceuticals (Canty et al. 2009; Dallas et al. 2013). In the present study, it was interesting to note very good correlations between induction of γ -H2AX foci with DNA strand breaks and micronuclei (Fig. 3.5). Such a relationship is increasingly recognized in mammalian in vitro studies (Yu et al. 2006). To our knowledge, such correlations between different genotoxicity parameters, especially in aquatic organisms have not been reported previously. The combined use of these biomarkers allows for holistic determination of the genotoxic damage induced by environmental agents which could be applied to other natural species.

One interesting aspect observed in this study is while no significant difference in terms of DNA damage is evident between the highest concentrations (32, 56 μg

L⁻¹, both species), there is a slight increase in γ -H2AX foci in the 56 $\mu\text{g L}^{-1}$ treatment. This could be a result of increased DSBs in the highest treatment, as opposed to less detrimental DNA lesions (i.e. SSBs) at lower Cu concentrations. γ -H2AX foci were present in the lowest Cu concentrations (18 $\mu\text{g L}^{-1}$), where the genotoxicity of Cu was not significantly apparent. Our data suggests that while DNA damage was not evident using the comet or MN assays, possibly due to lack of sensitivity, γ -H2AX is being recruited to damaged sites, in turn recruiting other DNA repair machinery even at low Cu concentrations. In light of this, γ -H2AX could be regarded as a more sensitive technique in measuring genotoxicity, however in this study, to a limited degree.

In terms of relative sensitivity of the comet and γ -H2AX assays, one major drawback of the comet assay is its inability to discriminate between DNA lesions, such as SSBs and DSBs, alkali labile sites and DNA interstrand crosslinks (Collins 2004, 8; Kumaravel and Jha 2006; Kumaravel et al. 2009; Liao et al. 2009). DSBs are considered to be most detrimental form of damage, they may be repaired or result in apoptosis and/or mutation. γ -H2AX is increasingly used as a biomarker in combination with classical and molecular techniques in mammalian systems (e.g. Yu et al. 2006; Oommen et al. 2016a, b). Whilst these techniques are considered to be simple and rapid (fast analysis time) in comparison to many other established methodologies (Liao et al. 2009), their relative sensitivity and effectiveness have not been compared sufficiently in ecotoxicological research. In this study, we compare the classical and novel techniques, examining their sensitivity as well as cost/time effectiveness. Although the biological damage measured by Comet and γ -H2AX assays are mechanistically different (one reflecting SSB/DSB, alkali labile site and another only DSB), the alkaline comet assay appears to be more suitable for larger

sample sizes that have high levels of DNA damage. On the other hand γ -H2AX, due to its high sensitivity and cost effectiveness, could be considered more suitable to smaller studies which aim to determine DNA damage at lower levels of exposure to those genotoxicants capable of effectively inducing DSBs, or contaminants which could induce DSBs at higher concentrations. This is particularly so as at higher levels of damage, induced foci can overlap making the scoring difficult and time-consuming. Overall, γ -H2AX has proved useful as a highly sensitive technique for detecting low-levels of DNA damage, its usefulness in ecotoxicological research, when combined with more classical techniques is clear.

Cu, as a model toxic metal is known to induce various types of damage to DNA and chromatin with potential pathophysiological consequences (Lloyd and Phillips 1999; Linder 2012). As mentioned earlier, one of the important mechanisms of induction of damage by Cu is via generation of ROS, inducing oxidative stress to biomolecules including DNA (Lloyd and Phillips 1999; Linder 2012). The modified comet assay using bacterial enzymes (e.g. FPG, Endo III) has been used by different workers to determine DNA oxidation in fish and mussels (Dallas et al. 2013; Mustafa et al. 2015). It would have been useful to determine DNA oxidation using modified comet assay in this study as well to determine relative contribution of DNA oxidation. This was however not feasible due to logistical problems. In addition, Cu in common with other toxic metals and metalloids (e.g. As, Co, Cd, Ni) could also interfere with DNA repair processes and cell cycle control (Hartwig 2013). In common with mammalian studies, elucidation of these fundamental processes in aquatic organisms following exposures to environmental contaminants also warrants attention. Interpreting

these highly conserved processes would help to further strengthen human and environmental links.

3.5 Conclusions

Our study has been the first to compare tissue specific accumulation and genotoxic effects following exposure to Cu in marine and freshwater bivalve gill cells. The zebra mussel, DP, is increasingly being utilised as a freshwater counterpart of *Mytilus* spp. in biomonitoring and ecotoxicological research. Our data highlights a clear relationship between external (water) and internal Cu concentrations. The capacity to concentrate contaminants within tissue makes MG and DP suitable bioindicator species to assess environmental health.

Cu induced comparable chromosomal and DNA damage in both mussel species, despite variable bioaccumulation of Cu into gill tissue. Furthermore, γ -H2AX foci formation was successfully applied as a useful biomarker of contaminant induced genotoxicity. The usefulness of this assay, particularly when applied alongside more classical, established techniques such as MN and comet assays is evident. While we cannot definitively associate the comparability in genotoxic response to differential species sensitivity, our results suggest that even low, environmentally realistic Cu concentrations have the potential to cause stress to some bivalve molluscs. For adequate protection of coastal and inland water bodies, future research would benefit from using a multi-species, multi-biomarker approach when investigating adverse effects at varying levels of biological organisation to gain a true understanding of the real environmental threat of the contamination to aquatic biota.

Chapter 4

Relative comparison of tissue specific bioaccumulation and radiation dose estimation in marine and freshwater bivalves following exposure to phosphorus-32



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4.1 Introduction

Short lived radionuclides such as ^{32}P (radiophosphorus), although occurring in small quantities in the environment may be capable of accumulating in aquatic biota (Smith et al. 2011). This is particularly so if the radionuclide is continuously discharged in the environment, and the biota is chronically exposed. In this context, ^{32}P is discharged into aquatic systems from various sources. For example, in England and Wales, 7, 5.2 and 5.7 GBq of ^{32}P was discharged in 2015 as liquid waste from educational, medical (i.e. hospitals) and other establishments (e.g. research, manufacturing and public sector) respectively (RIFE 2015). In terms of environmental concentrations, ^{32}P reference conditions in Scotland (i.e. concentrations that result in a total ingested dose for humans of 0.10 mSv y^{-1} if consumed at 2 L day^{-1}), are set at 57 Bq L^{-1} (DWQR 2014), with recorded values (2005-2013) averaging $0.27 \pm 0.21 \text{ Bq L}^{-1}$ in the River Clyde (Erskine Harbour, King George V Dock), Scotland (SEPA 2013). While not as environmentally relevant as radionuclides such as ^{137}Cs , ^{32}P was chosen due to ease of use in an experimental setting and as a surrogate for gamma emitting radionuclides, with sufficient penetrating energy to be detected outside the tissues of interest. In addition, phosphorus in the natural environment serves as an essential nutrient, and in common with non-radioactive phosphorus, radioactive phosphorus (^{32}P) is likely to have similar exposure pathways.

In terms of human health protection, contaminated organisms could pose a risk to health via the food chain (Jha 2004, 8; Aoun et al. 2015; Yang et al. 2015). ^{32}P uptake in humans may occur via dietary pathways, with dose being higher in the foetus and breastfed infants, than the adult (Oatway et al. 2008). Understanding radionuclide concentration patterns in biota allows for the development of adequate protection strategies, with the aim of reducing potential human dose

while maintaining environmental sustainability. Despite continuous and prolonged use in industry and subsequent discharges, no studies to our knowledge have investigated tissue specific accumulation of ^{32}P in aquatic biota. Bioaccumulative ability in aquatic bivalves, an important group of invertebrates of ecological and economic importance, has been identified in scientific literature. This is notably to ubiquitous, long-lived radionuclides such as ^{134}Cs , ^{210}Po , ^{210}Pb and ^3H (Evans 1984; Jha et al. 2005; Kalaycı et al. 2013; Feroz Khan et al. 2012, 2014; Dallas et al. 2016a; Metian et al. 2016; Pearson et al. 2018). However whole body accumulation and dose are often (but not always) the focus of such studies. Sufficient data are not available for tissue specific accumulation of short-lived radionuclides. It is well accepted that in common with other contaminants, radionuclides accumulate in the biota in a tissue specific manner. Whole-body determination of radionuclide bioaccumulation levels is important for risk assessments, however for biomonitoring and biological response studies (i.e. sensitive transcriptomics and proteomics studies), it is important that tissue specific information is generated. Radionuclide uptake disparity amongst tissues has been highlighted in studies from Jha et al (2005), Jaeschke et al (2011), Dallas et al (2016a) and Pearson et al (2018), where tritium accumulation in bivalve (*Mytilus* sp.) tissue was highly specific. Digestive gland (hepatopancreas/gut), gill and foot showed higher concentrations following exposure to varying amounts of tritium (5-15 MBq L⁻¹). Such trends are followed in green and brown mussels (*P. perna*, *P. indica*), where digestive gland showed maximum $^{210}\text{Po}/^{210}\text{Pb}$ activity over other biological soft tissue and shell (Feroz Khan and Godwin Wesley 2012). Furthermore in scallop (*Pecten maximus*) soft tissue, ^{241}Am was predominantly concentrated in the mantle and digestive gland, whereas ^{134}Cs was mainly present in the adductor muscle and mantle (Metian et

al. 2011). In environmental protection terms, understanding radionuclide accumulation is necessary to relate exposure, to radiation dose and potential biological responses. Exposure to IR can occur via multiple aqueous and dietary pathways, the behaviour and fate of radionuclides when accumulated in specific biological tissues or organs in the aquatic biota could be influenced by many factors and may vary significantly under different exposure scenarios (Pearson et al. 2018). Given that radionuclides accumulate differentially in the tissues, from a biomonitoring perspective, whole-body bioaccumulation monitoring is therefore not necessarily sufficient in fully protecting aquatic biota from the exposure. This is particularly important as differential tissue sensitivity could result in a detrimental biological response at levels presumed to be acceptable.

Dosimetry models, such as the Environmental Risk from Ionising Contaminants Assessment and Management (ERICA) Tool have been developed to evaluate radiological risk to aquatic and terrestrial biota (Brown et al. 2008). Risk is assessed by comparing a dose rate in a reference organism to a dose rate of $10 \mu\text{Gy h}^{-1}$ (0.24 mGy d^{-1}), a “screening dose rate” whereby no effect to populations of biota is expected (Garnier-Laplace and Gilbin 2006; Garnier-Laplace et al. 2008). Though dosimetry models are of great assistance in radiobiological research, ERICA tool predicted dose rates presume homogeneous radionuclide distribution within biota, which are represented as ellipsoidal shapes (Beresford et al. 2007). In order to adequately estimate radiological risk to biota, we require a greater knowledge of tissue specific radionuclide concentrations in a range of organisms, the transfer pathways, concentration factor, dose rate and an evaluation of any possible biological effects are required. Such data may also help pinpoint key tissues of interest for biomonitoring purposes.

The marine species *Mytilus galloprovincialis* (MG) and freshwater *Dreissena polymorpha* (DP) were selected in this study (Figure 1.4). The presence of radionuclides is of concern for both marine and freshwater environments. Although marine species might not be used to assess the risk in the freshwater environment or vice-versa, it is nevertheless important to estimate relative radionuclide accumulation in the biota belonging to same biological group. This would help to identify the most sensitive species for environmental protection. The present study had the following aims and objectives: (a) to determine tissue specific accumulation and depuration (release via excretion) of ^{32}P in two different species of mussels (i.e. marine and freshwater), (b) to evaluate the application of the ERICA tool in determining tissue specific radiation doses and (c) to identify the accumulation pattern of ^{32}P , as to highlight key tissues of interest for future experiments investigating biological response. It was hypothesised that whole body concentration of ^{32}P would be comparable in freshwater and marine bivalves, and that accumulation would be tissue specific.

4.2 Materials and methods

4.2.1 Chemicals and suppliers

Commercially available, radiolabelled-ATP (Adenosine triphosphate, γ - ^{32}P) was obtained from Perkin Elmer (PerkinElmer, UK) in batches of 9.25 MBq (specific activity: 370 MBq mL^{-1}) and used as the source of radioactive ^{32}P for our experimental purposes. Radiolabelled-ATP was diluted with DI water to form appropriate working solutions. Working solution added to beakers was decay adjusted. Nitric acid was obtained from Fisher Scientific UK (Nitric acid 68%, Primar Plus™) and scintillation cocktail from LabLogic systems Ltd. UK (ScintLogic, UK). All other chemicals and reagents were purchased from

Anachem Ltd. UK, Sigma-Aldrich Company Ltd UK, VWR International Ltd USA or Greiner Bio-One Ltd UK, unless stated otherwise. Additional product details are mentioned in text as appropriate.

4.2.2 Mussel exposure conditions

Two ten-day exposures were performed between December and February 2016-17. Adult MG and DP were collected and maintained in accordance to section 2.2. As Hilbish et al (2002) reported the occurrence and distribution of *Mytilus edulis*, *M. galloprovincialis* and their hybrids in the coastal regions of south-west England, we ensured species homogeneity in our experiments based on the method of Inoue et al (1995), in accordance to section 2.2.1.

Three MG and fourteen DP individuals per beaker (total weight of 35 g/beaker) were exposed to the following activity concentrations of ^{32}P in triplicate: 709, 7090 or 70900 and 571, 5710 or 57100 Bq L⁻¹, respectively, along with control treatments. The sample size (number of individuals) used in the study was decided to obtain a statistically robust set of data and was in line with previous studies (Dallas et al. 2016b). Activity concentrations in water were calculated from preliminary experiments (see section 2.8). Water changes (50 %) were carried out on days 3, 5, 7 and 9 and mussels were fed during this exposure (2 h before each water change), as described in detail elsewhere (Dallas et al. 2016a). Water samples (1 mL, in duplicate) were taken around 30 min after each water change and processed for liquid scintillation counting (LSC) to determine water activity concentrations.

Water quality parameters were measured routinely before and after water changes. Parameters were found to be within the expected range (pH 8.1 ± 1.2 , temperature 14.5 ± 1.8 °C, dissolved oxygen (DO) 96.9 ± 8 % and salinity $36.7 \pm$

0.6 for MG and pH 8.1 ± 0.3 , temperature 14.8 ± 0.9 °C, DO 92.3 ± 4.1 % and salinity 0.3 ± 0 for DP).

4.2.3 Sampling procedures and liquid scintillation counting

At the end of the exposure period, water from beakers was drained through a sieve (Fisherbrand, ISO 3310/1 250 µM). Faeces and pseudo faeces were collected from sieve on a weighed section of tissue, and placed into pre-weighed tubes (Punt et al. 1998; Jha et al. 2005). Mussels were dissected and separated into soft tissue (i.e. gill, mantle, adductor muscle, digestive gland and 'other' tissue), shell and internal mussel water (IMW). All samples were collected, processed and analysed as in section 2.8.3.1. Activity concentrations were background corrected by blank subtracting from each sample, the blank was non-spiked fresh or seawater. In accordance with Jaeschke and Bradshaw (2013), CPM values that fell below the blank were assigned an activity of 0.000. All samples were decay corrected.

4.2.4 Dosimetry and the ERICA TOOL

The Tier 2 assessment module of the ERICA tool was used for dose estimation. ^{32}P was chosen as one of the ERICA tool's default isotopes (Brown et al. 2008). Tissue specific dose rate (e.g. ^{32}P dose to digestive gland) was determined by taking mean measurements during sampling (i.e. mass, height, width, length), and developing custom geometry parameters on the ERICA tool (Table 2.4), the ERICA tool was utilised in accordance to section 2.8.4.

4.2.5 Statistical analysis

All values are mean \pm SD unless otherwise stated. Statistical analyses were performed in R (1.0.136; www.r-project.org). Data was checked for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test), the non-parametric Kruskal-Wallis test was used to evaluate effects of treatment on bioconcentration. Comparison between treatment groups was determined using a pairwise Wilcoxon test with Holm-Bonferroni correction. Level of significance for all tests was set at $p < 0.05$ unless otherwise stated.

4.3 Results

4.3.1 Activity concentrations in water

Activity concentrations in water (Table 4.1) showed good agreement with nominal values at 535, 6911 and 70253 Bq L⁻¹ for MG and 492, 4089 and 45611 Bq L⁻¹ for DP. Control water sample activities were below the LOD.

Table 4.1. Activity levels in water samples (Bq L⁻¹) per treatment in *M. galloprovincialis* and *D. polymorpha* (SD is standard deviation of mean data). Asterisks (*) denote nominal activity concentrations.

	Control	0.1 mGy d ⁻¹	1 mGy d ⁻¹	10 mGy d ⁻¹
MG*	0	709	7090	70900
MG	0.1 \pm 0.0	535.3 \pm 105.6	6911.4 \pm 1101.4	70252.8 \pm 5617.1
DP*	0	571	5710	57100
DP	0.1 \pm 0.0	492.1 \pm 279.0	4088.8 \pm 858.7	45611.1 \pm 9005.6

4.3.2 Activity concentrations in bivalve soft tissue, shell and IMW

In general, there appears to be a fairly high degree of variability between biological tissues (Fig. 4.1). Order of ^{32}P accumulation, in terms of total activity (Bq) per gram of tissue (wet weight), is illustrated in Table 4.2. Digestive gland showed the highest degree of accumulation over all treatments but DP control (Table. 4.2), independent of species. 87% (MG) and 45% (DP) of total activity within soft tissue is located in the digestive gland (10 mGy d⁻¹ treatment). MG digestive gland showed significantly higher values than DP across all treatments.

Table 4.2. Order of ^{32}P accumulation in soft tissue, shell and IMW in *M. galloprovincialis* and *D. polymorpha* individuals, order shows tissue with the highest to lowest bioconcentration (Bq g⁻¹) in all treatment groups.

Treatment (mGy d ⁻¹)	<i>M.galloprovincialis</i>				<i>D.polymorpha</i>			
	Control	0.1	1	10	Control	0.1	1	10
Highest	DG	DG	DG	DG	AM	DG	DG	DG
(Bq g ⁻¹)	Mantle	Gill	Other	Gill	Mantle	Shell	Other	Other
	Gill	Mantle	Gill	Other	DG	Gill	AM	Gill
	AM	Other	Mantle	Mantle	Gill	Other	Mantle	Mantle
	Other	AM	AM	AM	Other	Mantle	Shell	AM
	Shell	Shell	Shell	Shell	Shell	AM	Gill	Shell
Lowest	IMW	IMW	IMW	IMW	IMW	IMW	IMW	IMW

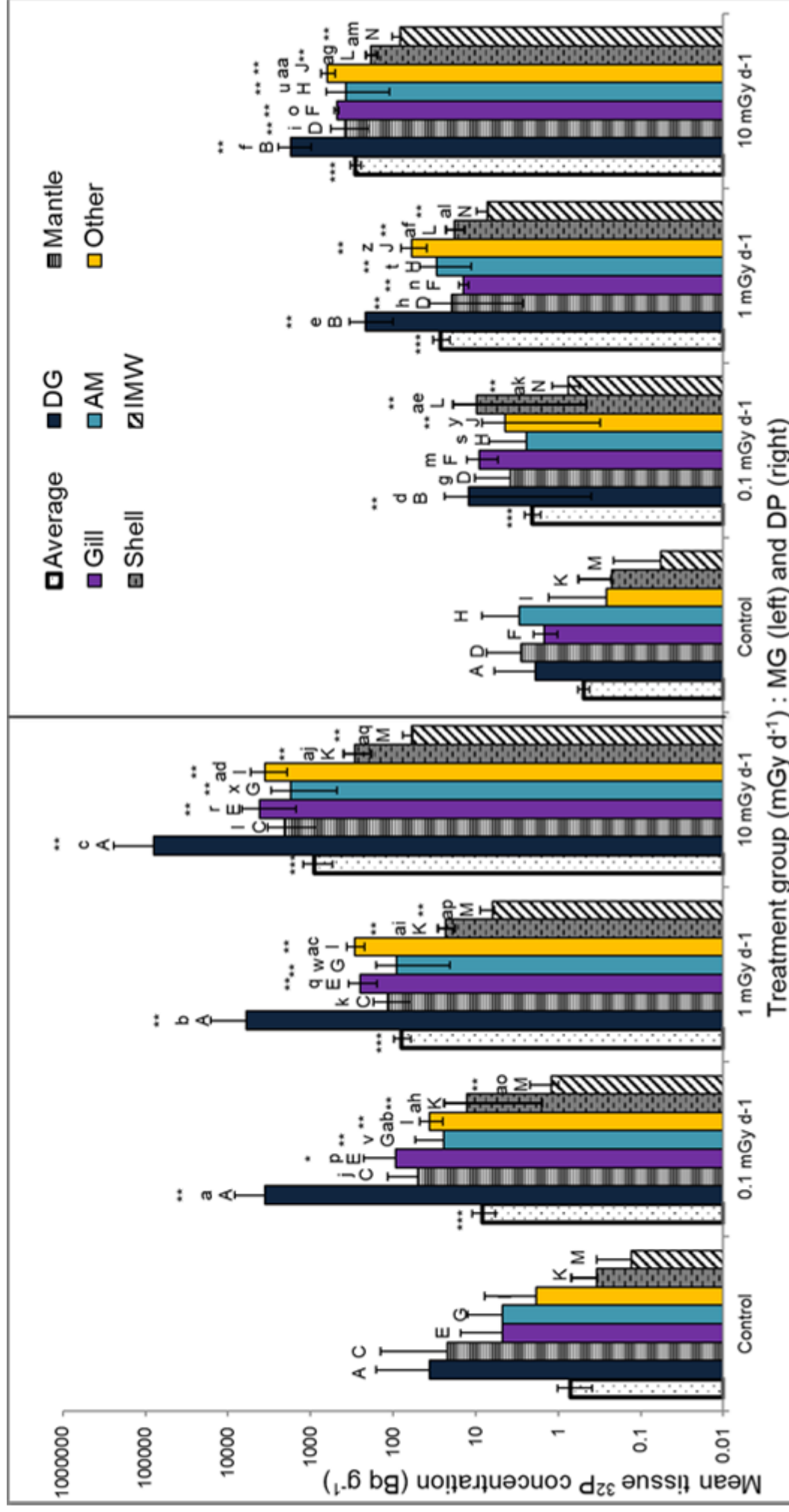


Figure 4.1. Tissue specific accumulation of ^{32}P in *M. galloprovincialis* (MG, left) and *D. polymorpha* (DP, right), total activity per gram of mussel tissue in control and irradiated treatment groups. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. Lower case letters denote variation between similar tissues (species specific, i.e. there is a significant difference in ^{32}P bioconcentration in digestive gland between each treatment group). Upper case letters denote significant variation in similar tissue and treatment group between species (e.g. MG digestive gland tissue values [0.1, 1 and 10 mGy d $^{-1}$ treatments] are significantly different than DP values in the corresponding treatment, there is no difference in control samples). SD is standard deviation of mean data. IMW – Internal mussel water.

4.3.2.1 Soft tissue

Apart from adductor mussel (AM) values between DP control and 0.1 mGy d⁻¹ ($p = 1$), bioconcentration increased in a dose dependant manner across all the tissues (Fig. 4.1), difference between treatments was not always statistically significant. In DP mantle and gill, no significance was noted between the control and 0.1 mGy d⁻¹ treatment ($p = 0.27$ and 0.16), this trend was not evident in MG mantle ($p < 0.01$). Mantle and gill values in 1 and 10 mGy d⁻¹ treatments showed a greater degree of ³²P activity than in controls and 0.1 mGy d⁻¹ treatments, independent of species. In all 'other' soft tissue, no variation is evident between species control ($p = 1$), or between the DP 0.1 mGy d⁻¹ and MG control treatments ($p = 0.11$). Proportions of ³²P in specific tissue are demonstrated on figure 4.2

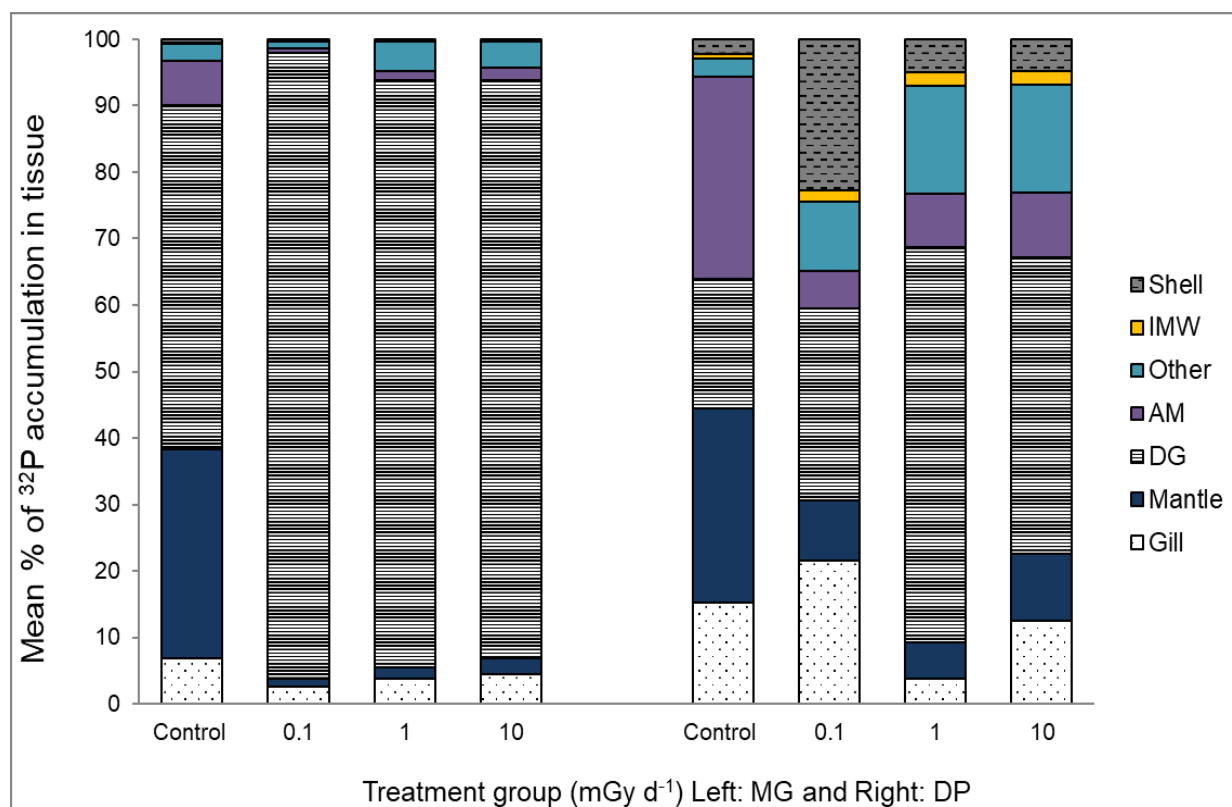


Figure 4.2. Proportion of ³²P in tissue after 10 day exposure in *M. galloprovincialis* (left) and *D. polymorpha* (right). IMW – Internal mussel water.

4.3.2.2 Internal mussel water (IMW) and shell

In all ^{32}P treatments, the lowest activity concentration was found in the IMW, followed by the shell (Table. 4.2). The activity concentration in IMW of both species is comparable to the nominal activity in water (Bq mL^{-1}). In terms of disparity between species, there is no significant difference in control samples ($p = 0.96$), this is also true between the DP 1 and MG 0.1 mGy d^{-1} ($p = 0.96$), and DP 10 and MG 1 mGy d^{-1} treatments ($p = 0.57$). There is a clear dose dependent response in the bivalve shell ($p < 0.01$), with the highest 10 mGy d^{-1} treatments showing the greatest activity concentration. From the 1 to 10 mGy d^{-1} treatment, there is an increase in total activity of 98% (MG) and 90% (DP). In terms of species comparison, there is no significant variance between shell bioconcentration in control treatments ($p = 0.1$).

4.3.2.3 Tissue specific ^{32}P accumulation

In terms of species, MG had a significantly higher degree of ^{32}P accumulation in all individual tissues ($p < 0.05$), for all the treatments. Bioconcentration of ^{32}P was more varied amongst DP tissue compared to MG. Proportionately (Fig. 4.2), in the 10 mGy d^{-1} treatment accumulation was as followed in MG; digestive gland (87 %)>gill (4.5 %)>other (3.9 %)>mantle (2.3 %)>adductor muscle (1.9 %)>shell (0.3 %)>IMW (0.1 %), and digestive gland (44.6 %)>other (16.2 %)> gill (12.5 %)>mantle (10 %)>adductor muscle (9.7)> shell (4.8 %) >IMW (2.1 %) in DP (Table 4.2).

4.3.2.4 Faecal matter and pseudofaeces

^{32}P release (Fig. 4.3) was determined by activity concentrations in faeces and pseudo-faeces. Due to the experimental set-up it was not feasible to distinguish between the two. In both species, activity concentrations (Bq g^{-1} faeces) rise in a dose dependent manner ($p < 0.001$). Concentrations of ^{32}P in faeces and pseudo-faeces from the 10 mGy d^{-1} treatment was significantly higher than in all treatments ($p < 0.001$), with DP faeces having the greatest total activity at 625.1 Bq g^{-1} compared to 466.1 Bq g^{-1} . There is, however, no statistical variation ($p = 0.2$). Both species independently displayed significant differences between radioactive treatments, but no variation was seen between species; 0.1 mGy d^{-1} ($p = 0.9$), 1 mGy d^{-1} ($p = 0.09$) and 10 mGy d^{-1} ($p = 0.2$). No variance was observed in control bivalves.

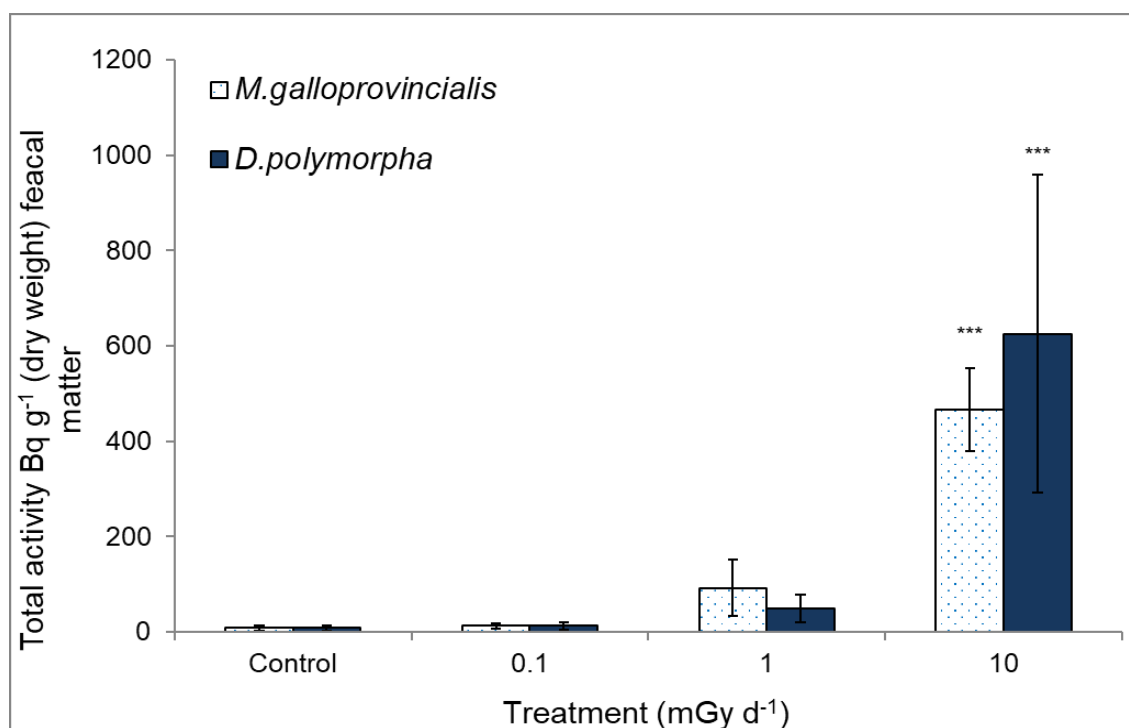


Figure 4.3. Activity levels (Bq g^{-1}) in *M. galloprovincialis* and *D. polymorpha* faecal matter (dry weight), following ^{32}P exposure. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01 , 0.001) from the corresponding control. SD is standard deviation of mean data.

4.3.3 Dosimetry

Tissue activity concentrations of ^{32}P reached $41\pm3\%$ of the value of the surrounding water in MG, as opposed to $17\pm3\%$ by DP. Using tier 2 of the ERICA tool, the average achieved total body dose rates were calculated to be 0.07, 0.68 and 7.25 mGy d^{-1} for MG, and 0.02, 0.24 and 2.62 mGy d^{-1} for DP, falling short of the expected values of 0.1, 1 and 10 mGy d^{-1} (Table 4.3). Table 4.3 demonstrates water activity concentrations that give the correct dose (Bq L^{-1}), corrected to 35 g whole mussel tissue (Inc. soft tissue, shell and IMW)/beaker. Whole mussel tissues (i.e. soft tissue, shell and IMW), as opposed to just soft tissue, were used in dose rate calculations as to more accurately reflect internal dose rate. This is particularly important for future experiments where biological effects are determined in mussel species.

Table 4.3. Table to show (a) the expected dose rates in mGy d^{-1} and $\mu\text{Gy d}^{-1}$ (for the ERICA tool), (b) the water activity concentrations that give the correct dose rate (Bq L^{-1}) for both species *as calculated from preliminary experiments, (c) the average dose rate achieved in mGy d^{-1} and (d) ERICA tool water activity concentrations that give the correct dose (Bq L^{-1}), corrected to 35 g whole mussel weight/beaker.

		Expected dose rate mGy d^{-1}	$\mu\text{Gy d}^{-1}$ (ERICA)	ERICA tool water concentrations that give correct dose rate (Bq L^{-1})*	Average Dose rate mGy d^{-1}	ERICA tool water concentrations that give correct dose rate (Bq L^{-1}) - 35 g
MG	0.1		4.17	709	0.07	993
	1		41.7	7090	0.68	9930
	10		417	70900	7.25	99300
DP	0.1		4.17	571	0.02	2250
	1		41.7	5710	0.24	22500
	10		417	57100	2.62	225000

In consideration to the significant degree of accumulation found in the digestive gland, independent of species, a tissue specific dose rate was calculated using the ERICA tool. Dose was determined by creating two new organisms; *D. polymorpha* (DG) and *M. galloprovincialis* (DG), occupancy factors and tissue specific organism geometry are listed in Table 2.4. Input parameters were mean measurements taken from experimental samples from bioaccumulation experiments. The average achieved dose rates in digestive gland were calculated to be 20.76, 35.28 and 468 mGy d⁻¹ for the MG, and 0.07, 1.16 and 9.22 mGy d⁻¹ for DP (Table 4.4).

Table 4.4. Table to show the expected and achieved dose rates (mGy d⁻¹) in *M.galloprovincialis* and *D.polymorpha* digestive gland using custom geometry in the ERICA tool (Tier 2)

	Expected dose rate mGy d ⁻¹	Av. Achieved dose rate mGy d ⁻¹
<i>M.galloprovincialis</i>	0.1	20.76
	1	35.28
	10	468
<i>D.polymorpha</i>	0.1	0.07
	1	1.16
	10	9.22

To confirm and validate data analysis using the ERICA tool, tissue specific dosimetry calculations were compared to data showing total activity per gram of tissue (Bq g⁻¹). In MG, there was a 41% and 92% increase between the 0.1 and 1, and 1 and 10 mGy d⁻¹ treatment groups in both activity concentrations in tissue (Bq g⁻¹) and dose rate. In DP, there was a 94% and 87% increase between the 0.10 and 1, and 1 and 10 mGy d⁻¹ treatment groups in both activity concentrations in tissue (Bq g⁻¹) and dose rate.

Concentration factor values, calculated by dividing tissue specific ^{32}P activity concentrations (Bq kg^{-1} , wet weight) by activity concentrations of the spiked water, were as follows; 11.7, 11.2 and 11.9 in MG and 3.6, 4.6 and 5 for DP (in 0.1, 1 and 10 mGy d^{-1} treatments).

4.4 Discussion

From this study, it is evident that ^{32}P accumulation is highly tissue specific and variable between bivalve species. It is important to note that in this study ^{32}P was introduced in a highly bioavailable form (i.e. radiolabelled ATP), demonstrated bioaccumulation patterns in this study may be reflective of this. MG, which accumulated $41\pm3\%$ of ^{32}P present in the surrounding media as opposed to $17\pm3\%$ by DP, showed a greater degree of ^{32}P accumulation across all biological tissues. Despite variance in uptake and accumulation, ^{32}P excretion was comparable between species. It could be assumed that the measured activity concentration takes into account absorption, metabolism of ATP, subsequent dispersal and partitioning of phosphorus in tissue specific manner at a given sampling time. This phenomenon as a whole could be considered as tissue specific accumulation of radiophosphorus. It is also possible that the tissues could have achieved equilibrium over the exposure period. It would, however, be difficult to predict tissue dose delivered by the available radionuclide concentration in the surrounding media. Furthermore, equilibrium status is often regarded as a flaw in the ERICA tool. In terms of dosimetry, the ERICA tool proved valuable in calculating whole body and tissue specific dose rates. Average achieved dose rates were 0.07, 0.68 and 7.25 mGy d^{-1} for MG, and 0.02, 0.24 and 2.62 mGy d^{-1} for DP, below expected values of 0.10, 1 and 10 mGy d^{-1} . The

dose dependant nature of ^{32}P accumulation gives evidence that both marine and freshwater bivalves are suitable bioindicators of radioactive pollution.

In consideration of species, MG accumulated a higher degree of ^{32}P in biological tissue across all treatments. Such disparity may be a result of several biotic and abiotic variables, including physiology (filtration rates, metabolism, and reproductive stage), biochemistry and water chemistry (salinity, pH, dissolved oxygen, radionuclide speciation) (Nalepa et al. 1991; Reinfelder et al. 1998; Konovalenko et al. 2016; Pearson et al. 2018). The underlying mechanism which leads to differences between freshwater and marine bivalves is unclear, differential radionuclide accumulation between bivalves is a topic with little attention to date. In terms of stable phosphorus (P), tissue bioconcentration has been found to vary dependent on reproductive processes, high P concentrations are evident during periods of spawning in *Mytilus* sp. and DP (Kuenzler 1961; Jurkiewicz-Karnkowska 2002). Jurkiewicz-Karnkowska (2002) noted variability in soft tissue P concentrations between three freshwater bivalves (DP, *Anodonta anatina* and *A. cygnea*) inhabiting the Zegrzynski Reservoir, Poland, suggesting species specificity in terms of stable P accumulation.

Feeding and digestion is often regarded as a predominant route of radionuclide intake (McDonald et al. 1993). The digestive gland in bivalves plays a central role in metabolism. It is important for intracellular digestion, as a storage site for metabolic reserves during periods of stress, and as a site of nutrient distribution to other organs, particularly reproductive tissue (Cartier et al. 2004). Under all treatment groups the greatest ^{32}P concentration was present in the digestive gland, at 87% in MG and 45% in DP of the total activity within soft tissue (10 mGy d^{-1}), suggesting a dietary route of exposure. The findings are supported by earlier studies by Jaeschke et al (2011) and Jha et al (2005) who reported preferential

tritium accumulation in *Mytilus* sp. digestive gland (tritiated glycine, 1.48 MBq L⁻¹ and tritiated water, 3.7, 37 and 147 MBq L⁻¹). This trend is continued in *Mytilus* spp., following exposure to ²⁴¹Am, ³²⁹Pu, ²³⁷Np and ⁶³Ni, (McDonald et al. 1993; Punt et al. 1998) and in marine amphipods exposed to ³²P (Johannes 2003). Variance between marine and freshwater bivalves may result from differential physiological and genetic characteristics. In terms of physiology, filtration rates have been noted as comparable between species, at 76.6 (DP) and 87.5 mL mussel⁻¹ h⁻¹ (*Mytilus edulis*, ME), along with valve movement at 90.1 (DP) and 92 (ME) % of open valves under ambient conditions (Rajagopal et al. 2003). While neither parameter were measured in this study; it is possible that DP individuals are more inclined to close their valves when exposed to ³²P, as a stress response. This behaviour is documented in biofouling control research, where bivalves close valves during periods of water chlorination as a protective strategy (Rajagopal et al. 2003). Observed differences may also result from variable feeding regimes (i.e. species fed different food types) and/or gut physiology. Factors involved with digestion such as food density or quality, gut passage time, volume or retention rate, enzymatic composition, digestive partitioning and chemistry may effect ³²P assimilation (Wang et al. 1995). Despite the disparity in CF values between MG and DP, the trend between biological tissues is similar, suggesting comparability in ³²P accumulation pathways.

Average achieved dose rates in digestive gland were calculated at 20.76, 35.28 and 468 mGy d⁻¹ for MG, and 0.07, 1.16 and 9.22 mGy d⁻¹ for DP. This specific tissue dose is substantially greater than calculated whole body doses, suggesting that whole-body dose monitoring may be insufficient in wholly protecting aquatic organisms from radionuclide exposure. Tissue specificity, in terms of accumulation is well documented for many radionuclides. Strontium-90 for

example is a 'bone seeker', due to its biochemically similar behaviour to calcium, following ingestion a large proportion will attach to the surface, or be absorbed into bone (ATSDR 2004). In the context of biomonitoring and adequate environmental protection, an understanding of tissue specific dose rates is of high importance. Calculated whole body dose for MG and DP (0.1 and 1 mGy d⁻¹ treatments) fell below the predicted 'no effects' screening value of 10 µGy h⁻¹ (0.24 mGy d⁻¹), suggesting a minimal risk to the individual or population. However, in all but the DP 0.1 mGy d⁻¹ treatment, digestive gland dose was above the screening benchmark.

In the 10 mGy d⁻¹ treatment, gill tissue had 1816% (MG) and 255% (DP) less concentrated ³²P than in the digestive gland (Bq g⁻¹). As filter feeding organisms, particulates within the water column are captured within cilia on the gills, particulate matter is then carried via mucous strings to the mouth (Riisg et al. 2011). Gill tissue may therefore act as a major pathway for contaminants to enter other biological tissue. IMW activity concentrations are comparable to the expected activity in water (Bq mL⁻¹) in both species, suggesting that bivalves are unable to regulate ³²P uptake via aqueous pathways. Past studies have often highlighted gill as a tissue of key concern due to proximity to the surrounding media, high surface area and water content. The relatively low activity in *Mytilus* sp. gill tissue relative to the digestive gland is a trend found in other studies following exposure to tritium (12 to 485 µGy h⁻¹) and nickel (⁶³Ni) (Punt et al. 1998; Jha et al. 2005). In terms of subsequent biological response, it is important to note that while ³²P may have accumulated to a lesser degree in some tissue, the beta emission can penetrate approximately 0.76 cm of tissue/water (Terrance 2017). By proximity, higher dose rates may be evident in tissue or cells not directly accumulating ³²P to a high degree. In terms of gill tissue, while a relatively

low contaminant concentration is observed, its large surface area and proximity to surrounding media may result in a higher absorbed dose.

In the natural environment, many factors may influence the filtration rate of bivalves, along with feeding and depuration rate. Changing environmental factors, such as water quality conditions, food availability, reproduction and physiological condition may affect feeding behaviour (Riisg et al. 2011). Laboratory conditions may not accurately reflect feeding, and therefore uptake and depuration patterns of ^{32}P in bivalves may vary. It is also possible that due to different habitats, certain bivalve species are either more adapted to, or have experienced more disturbances or stresses in the wild, and are therefore more resilient to stresses under laboratory conditions. However relative response to a particular stressor of similar magnitude in two different species, representing different habitats, is difficult to estimate in the natural environment. From an environmental protection perspective, an understanding of radionuclide transfer pathways under environmentally realistic conditions, whether uptake is dietary (ingestion of contaminated food) or through direct transfer from surrounding media is important. One of the limitations of the study is that these laboratory-based experiments were carried out in static exposure conditions, which differs from real environmental situations. A flow-through exposure set-up would have been a more realistic experimental design but due to health, safety, logistics and economic reasons (requiring large amounts of radionuclides), a flow-through experimental design was not feasible. Further studies using a wider range of radionuclides and exposure conditions, which better reflect environmental exposure conditions (e.g. flow through system) would be of great benefit. Knowledge of the behaviour and transfer of radionuclides within aquatic systems

allows for an assessment of potential impacts and subsequent management strategies.

Understanding excretion of contaminants is important firstly as a means of determining possible chronic effects of assimilated contaminants, and secondly in respects to human consumption. In terms of public health, depuration is mandatory in bivalves harvested for human consumption as to remove contaminants, predominantly bacteria (Lee et al. 2008). The effectiveness of depuration in removing radionuclides is yet to be fully understood. Suspension feeding bivalves produce faeces and pseudofaeces, the latter of which refers to particles rejected before entering the gut. Excretion of ^{32}P , measured in a combination of faeces (from alimentary tract) and pseudofaeces (from mantle cavity), do not appear to be consistent with that observed from uptake. In irradiated treatments (0.10, 1 and 10 mGy d⁻¹), 0.31%, 0.15% and 0.08% (MG) and 0.4%, 0.15%, 0.34% (DP) of ^{32}P from surrounding media was excreted; significantly lower than the 41±3% (MG) and 17±3% (DP) of ^{32}P accumulated within biological tissue. While our findings suggest a slow depuration rate during IR exposure, results are limited in showing a brief snapshot in time. It would be of interest to monitor uptake and excretion, and therefore depuration rates over both a longer duration, and following the removal of ^{32}P in water.

The shell surface of aquatic bivalves is known to adsorb dissolved contaminants from surrounding media (Zuykov et al. 2012), thus why in this study whole body dose was not limited to just soft tissue. When removing both IMW and shell concentrations from the data before ERICA tool analysis, the results follow exactly the same pattern due to the influence of vast ^{32}P concentrations in the digestive gland. ^{32}P biosorption in whole shell was concentration dependant in both species, with an increase in total activity of 98% (MG) and 90% (DP)

between 1 and 10 mGy d⁻¹ treatments. Proportionately DP showed higher incorporation into shell, over all treatments, whereas per gram of shell, MG has significantly greater ³²P present. Mollusc shell is formed of a few calcified layers and the periostracum, one thin, organic coating layer (Marin et al. 2012; Zuykov et al. 2012). Species variation may be a result of differing shell microstructure and topography, chemical and macromolecule composition (Marin et al. 2012). As noted by Zuykov et al (2012), MG and DP do show disparity in shell topography, where DP has a thinner periostracum and a lamellate surface (Immel et al. 2016). In this study, the content of ³²P in shell was far lower than in soft tissue, this data contrasts to findings by Koide et al (1982), Clifton et al (1989) and Metian et al (2011) following exposure to radionuclides or heavy metals. As an example, in scallop (*Pecten maximus*), biosorption of ²⁴¹Am into the shell was far greater than soft tissue, however in the same species, ¹³⁴Cs showed preferential accumulation in soft tissue over shell (Metian et al. 2011). Bivalve shells are widely used to monitor pollutants in the aquatic environment (Zuykov et al. 2013). It is relevant to note that bioconcentration values taken from shell are not reflective of soft tissue values.

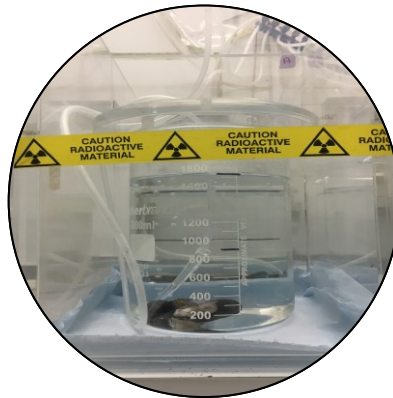
4.5 Conclusions

This is the first study to compare uptake and depuration (via excretion) of short-lived radionuclide, ³²P in two anatomically similar bivalve species. Accumulation of ³²P is highly tissue specific, with the majority located within the digestive gland. This is particularly important in the context of biomonitoring and adequate environmental protection, where whole-body dose monitoring may not always be sufficient to protect aquatic organisms from radionuclide exposure. Differential sensitivity between biological tissues could result in harmful biological response

at activity levels presumed to be safe. The next step is to link radioactive exposure, accumulation and dose rate, to consequent biological responses (chapter 5). Accumulation within mussel tissues, even for short durations may potentially have long lasting effects in both exposed individuals and subsequent generations. Lastly, considering species variation in ^{32}P accumulation, it is not necessarily accurate to evaluate accumulation or biological hazard of ionising radiations to the marine environment by using information gathered from freshwater systems, and vice versa.

Chapter 5

Assessing relative biomarker responses in marine and freshwater bivalve molluscs following exposure to phosphorus 32 (^{32}P): Application of genotoxicological and molecular biomarkers



In preparation:

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5.1 Introduction

IR emitted from radionuclides discharged in the environment can potentially pose short and long-term detrimental effects to both human and non-human biota, with DNA being the most important target for their actions (UNSCEAR 1982; Dallas et al. 2012). A generic (all species) “no effect” dose rate of $10 \mu\text{Gy h}^{-1}$ (0.24 mGy d^{-1}) has been adopted as a screening value, dose rates under this value are thought to result in minimal risk to the individual or population of natural species (Andersson et al. 2008, 2009). There is, however, not enough experimental information available in the literature to support this generic ‘no effect’ dose rate for wider natural biota (Dallas et al. 2012). To ensure an adequate degree of protection, therefore, there is a necessity to link radiation exposure to tissue specific bioaccumulation and dose rate, and to subsequent biological responses in a range of aquatic organisms to determine their relative sensitivity (Scoppa 1983; Dallas et al. 2012; Kumar et al. 2017; Carvalho 2018; Salbu et al. 2018; Skipperud and Salbu 2018; Vernon et al. 2018).

As highlighted in section 1.3.2, molecular and genetic alterations are perceived as an early warning signal of organism’s stress. Exposure to IR has the potential to cause short and long term damage to aquatic organisms at different trophic levels including invertebrates and fish, this has been demonstrated by the detrimental biological responses caused by widespread, long-lived, radionuclides such as ^{137}Cs and ^3H (Walker et al. 2000; Jha et al. 2006; Olsvik et al. 2010; Farcy et al. 2011; Freeman et al. 2014; Dallas et al. 2016a; Arcanjo et al. 2018; Hurem et al. 2018; Pearson et al. 2018).

The majority of studies aiming to determine potential detrimental effects of IR have focused primarily on long-lived radionuclides. However, short-lived radionuclides such as ^{32}P , whilst occurring in small quantities within the

environment have the capacity to accumulate in aquatic biota, particularly when they are chronically exposed (Smith et al. 2011). Once concentrated in tissues, the radioisotope has the potential to induce significant molecular and genetic level effects. Phosphorus-32 is chemically and radiologically unique, the mode of action (MoA) is mediated by induction of DNA DSBs (Cheng et al. 2015). Aqueous ^{32}P gets incorporated into the ribose-phosphate backbone of replicating DNA, isotopic decay (^{32}P to sulfur-32, ^{32}S) results in chemical breakage of DNA (SSBs), and the release of high energy beta particles causes further DNA damage through DSBs (Cheng et al. 2015). While not as environmentally prominent as radionuclides such as ^{137}Cs or ^{90}Sr , ^{32}P can be utilised as a relatively cheap, easy to use (in terms of experimental design) surrogate for beta and gamma emitting radionuclides (Vernon et al. 2018).

Given that DNA is the most important target for the actions of IR, several studies have been carried out to determine its impact on DNA using a range of endpoints (e.g. induction of DNA strand breaks, MN and chromosomal aberrations) in aquatic species (Dallas et al. 2012). γ -H2AX foci induction following ^{32}P exposure (111 kBq, 24 h) has been displayed in HeLa S3 cells, mouse BALB/c CRL2836 cells and other malignant cell lines, along with other biological responses (Cheng et al. 2015; Oommen et al. 2016a, 2016b). Aqueous $^{32}\text{P}[\text{PO}_4]$ has also been investigated as a possible novel anti-cancer drug (Cheng et al. 2015). In terms of aquatic biota, the use of this DDR biomarker (i.e. γ -H2AX) is somewhat limited, and to date has only been utilised in fish (*Danio rerio*, *Pimephales promelas*, *Oryzias latipes*) cells (Choi et al. 2010; Pereira et al. 2011; Gagnaire et al. 2017; Sayed et al. 2017; Si et al. 2017).

With respect to IR induced mRNA alterations, there is limited information available for aquatic invertebrates (Farcy et al. 2007, 2011; AlAmri et al. 2012;

Han et al. 2014a,b; Devos et al. 2015; Dallas et al. 2016a, Gomes et al. 2018). In *Mytilus* sp., genes involved with protein folding, DNA DSB repair and cell cycle checkpoint control (e.g. *hsp70*, *hsp90*, *mt20*, *p53*, *rad51*) were altered at dose rates of 15.13-18.49 $\mu\text{Gy h}^{-1}$ (72 h, tritium, Dallas et al. 2016a). Similarly, AlAmri et al (2012) noted altered *rad51* mRNA expression levels, a gene involved in DSB repair, in *M. edulis* exposed chronically to low dose rates of 0.61 $\mu\text{Gy h}^{-1}$. In contrast, the expression of key stress genes (i.e. *hsp70*, *hsp90*, *hsc72*, *gst*, *mdr*, *cyp1a*, *sod*, *mt1&2* and *p53*) in oysters (*C. gigas*) larvae and spat exposed to chronic dose rates of 29.3 and 27.4 mGy h^{-1} (14 d, ^{137}Cs , ^{241}Am), respectively, remained unchanged in comparison to controls (Devos et al. 2015). This was paralleled for the 9 target genes in *C. gigas* gill tissue following a 14-day exposure to tritium ($\sim 15\text{-}18 \mu\text{Gy h}^{-1}$, Devos et al. 2015). Such disparity in data could be attributed to differential tissue and species sensitivity to IR, physiological factors such as reproductive stage, age or general health status of the species or potentially due to differences in experimental design including sources and exposures of IR used (Jha 2008; Devos et al. 2015). In common with mammalian studies, evidence suggests that aquatic invertebrates are vulnerable to IR-induced damage at a molecular level. More studies are nevertheless required as the limited amount of information available in the literature have evaluated expression of genes and other biological responses following exposure of organisms to external radiation sources, which could not be considered environmentally relevant (Dallas et al. 2012).

In the background of above information and due to (a) the nature of ^{32}P , (b) its environmental relevance for both freshwater and marine environments and (c) limited amount of information available with respect to its potential impact on natural biota, in this study, we aimed to investigate genetic and molecular

alterations in two ecologically relevant bivalve species. Adopting a multi-genotoxicological biomarker approach, we aimed to investigate IR-induced damage, incorporating both classical (Comet and MN assays) and novel techniques (i.e. γ -H2AX induction), along with transcriptional responses of key genes to assess broader responses, in MG and DP individuals. The overall aims and objectives of this study are: (a) to adopt a multi-biomarker approach to establish genotoxic and molecular responses in two bivalve species following ^{32}P exposure, (b) to determine relative sensitivity of marine and freshwater adult bivalves and (c) to link accumulation and radiation doses to subsequent biological responses, in gill and digestive gland tissues. In terms of variation between bivalve species subsequent to ^{32}P exposure, we hypothesised that little disparity in genotoxic or molecular response would be evident, increased DNA damage would be paralleled by DDR, and lastly, genes related to oxidative stress would be upregulated following ^{32}P exposure.

5.2 Materials and methods

5.2.1 Chemicals and suppliers

Commercially available, radiolabelled-ATP (Adenosine triphosphate, γ - ^{32}P), was obtained from Perkin Elmer (PerkinElmer, UK) in batches of 9.25 MBq (specific activity: 370 MBq mL^{-1}), and diluted with DI water to form appropriate working solutions. Working solution added to beakers was decay adjusted.

5.2.2 Mussel exposure conditions

Two ten-day exposures were performed in June and September 2017 (Fig. 5.1). DP and MG were maintained in accordance to Chapter 2, section 2.2.

Exposures of mussels to ^{32}P and positive control (Cu) were staggered by one week for ease of analysis and logistical reasons. MG and DP individuals (total wet weight, 35 g L^{-1}) per labelled beaker were exposed to the following activity concentrations of ATP [$\gamma\text{-}^{32}\text{P}$] in triplicate: 993, 9930, 99300 and 579, 5786, 57860 Bq L^{-1} , respectively, to meet the expected dose rates of 0.10, 1.00 and 10 mGy d^{-1} . Nominal ^{32}P activity levels in water were calculated from preliminary experiments (chapter 4, Vernon et al. 2018). A negative control and positive control (copper [Cu], as $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 99% purity, $56 \mu\text{g L}^{-1}$) were run alongside. Water changes (50 %) were carried out on days 3, 5, 7 and 9. ^{32}P activity levels were determined using water samples (1 mL, in duplicate), taken ~30 minutes after each water change and processed for liquid scintillation counting (LSC) as in section 2.8.3.2. Mussels were fed 2 h prior to water changes as described in earlier studies (Vernon et al. 2018). Water quality parameters (i.e. pH, temperature, salinity and DO) were measured routinely, before and after water changes. ^{32}P and Cu concentrations were determined as per standard procedures (described in sections 2.7.2 and 2.8.3), along with water quality measurements, which are displayed in Table 5.1. Data from LSC counting and ICP-MS analysis confirmed that achieved values were in line with expected concentrations across all treatments.

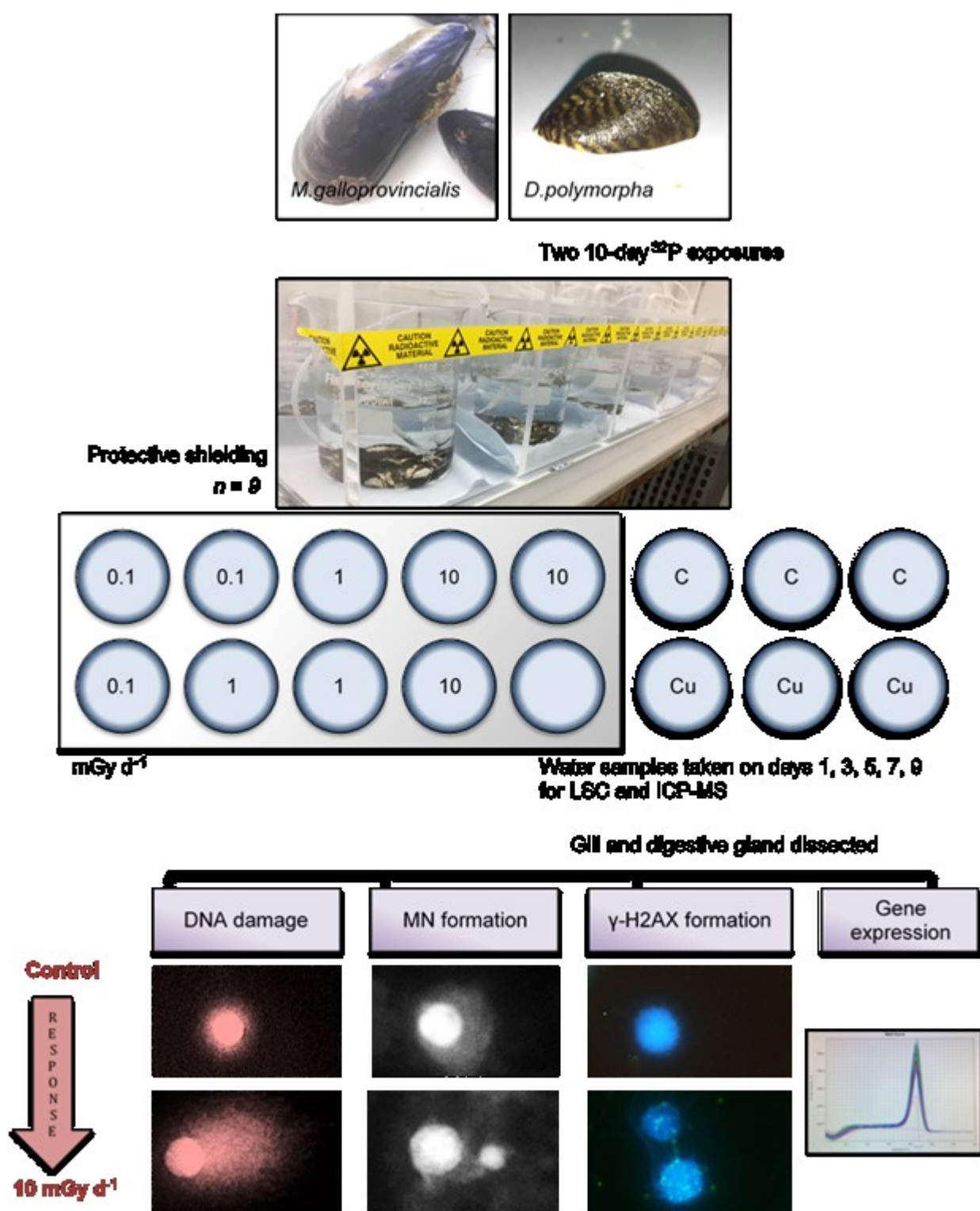


Figure 5.1. Schematic diagram showing experimental design. Images from *M. galloprovincialis* digestive gland cells.

Table 5.1. Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO], average taken from daily measurements), copper concentrations in water ($\mu\text{g L}^{-1}$) and ^{32}P concentrations in water (Bq L^{-1}), for both species. Data are presented as mean \pm standard deviation.

Water parameters	<i>M. galloprovincialis</i>	<i>D. polymorpha</i>
pH	8.1 \pm 0.96	8.2 \pm 0.18
Temp ($^{\circ}\text{C}$)	14.8 \pm 0.16	14.8 \pm 0.16
Salinity	33.7 \pm 0.34	0.3 \pm 0.01
DO (%)	99.7 \pm 1.14	100.5 \pm 0.85
Copper water conc. ($\mu\text{g L}^{-1}$)		
Control	0.1 \pm 0	0.1 \pm 1
56	43.5 \pm 8.52	59.7 \pm 3.74
^{32}P water conc. (Bq L^{-1})		
Control	0.2 \pm 0.60	0.3 \pm 0.44
993 / 289	1032.2 \pm 327.63	263.9 \pm 95.85
9930 / 2892	9916.1 \pm 1120.82	2687 \pm 395.37
99300 / 28928	98716.7 \pm 6429.27	26561 \pm 2776.52

5.2.3 Sampling procedures

Subsequent to exposures, gill and digestive gland tissues were dissected from each individual as described in detail in earlier studies from our laboratory (Dallas et al. 2013; Pearson et al. 2018; Vernon et al. 2018). Dissected tissues were stored as followed until use: $\frac{2}{3}$ tissue stored in tube on ice until cell isolation, $\frac{1}{3}$ stored in RNeasy lysis buffer (1.5 mL, Fisher UK) at -20°C . All other soft tissues and shell were discarded.

5.2.4 Biological assays

5.2.4.1 Isolation of digestive gland and gill cells

The procedure to obtain gill cells for genotoxic assays was adopted from previous studies (Vincent-Hubert et al. 2011), and outlined in section 2.3.2. Supernatant was then used in subsequent assays, providing cell viability, checked using the Trypan Blue exclusion dye assay (section 2.4) (Strober 2001), was <90% across all treatments (data not shown).

5.2.4.2 Comet assay to determine DNA strand breaks

The comet assay was performed using gill and digestive gland cell suspension (150 μ L), as described in section 2.5.2.

5.2.4.3 Analysis of micronuclei (MN) induction

Gill and digestive gland cell suspension was adhered and fixed as described in section 2.5.1, before staining with 20 μ L ethidium bromide (20 μ L of 20 mg L⁻¹). Cells ($n = 500$) were scored per slide, and results are reported as mean MN per 1000 cells, in keeping with other data from our research group (Dallas et al. 2013).

5.2.4.4 Induction of γ -H2AX foci

γ -H2AX foci were determined in gill and digestive gland cells, following procedures outlined in 2.5.3. All slides, including procedural blanks were coded and scored at random, and 50 cells were counted per individual/slide.

5.2.4.5 Determination of transcriptional expression of key genes

Gill and digestive gland tissue was dissected immediately after exposure and stored in RNAlater at -80 °C (R0901, Sigma-Aldrich Company Ltd UK) until use. RNA extraction, cDNA synthesis and qPCR were performed in accordance to sections 2.5.4.1 – 2.5.4.3.

5.2.5 Determination of ^{32}P and Cu concentration in water samples

5.2.5.1 Determination of ^{32}P in water samples using liquid scintillation counting

All samples were collected, processed and analysed as in section 2.8.3.1. Activity concentrations were background corrected by blank subtracting from each sample, the blank was nonspiked fresh or seawater. In accordance with Jaeschke and Bradshaw (2013), CPM values that fell below the blank were assigned an activity of 0.000. All samples were decay corrected.

5.2.5.2 Determination of Cu concentration in water samples using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Cu concentrations in water samples were determined following procedures outlined in section 2.7.2.

5.2.4 Dosimetry and the ERICA tool

The Tier 2 assessment module of the ERICA tool was used for dose estimation. Phosphorus-32 was chosen as one of the ERICA tool's default isotopes (Brown et al. 2008).

Tissue specific dose rate (e.g. ^{32}P dose to digestive gland) was determined by taking mean measurements during sampling (i.e. mass, height, width, length), and developing custom geometry parameters on the ERICA tool (Table 2.4), the ERICA tool was utilised in accordance to section 2.8.4. Dose rates for whole-body, gill and digestive gland tissue are presented in Table 5.2.

Table 5.2. Table to show (a) the expected dose rates in mGy d^{-1} and (b) the average dose rate achieved in *M. galloprovincialis* and *D. polymorpha* whole-body, digestive gland and gill tissue (mGy d^{-1}).

	Expected dose rate	Av. Dose rate (mGy d^{-1})		
		Whole body	Digestive gland	Gill
<i>M. galloprovincialis</i>	0.1	0.10	4.32	0.06
	1	0.97	39.12	0.78
	10	10.66	420	10.13
<i>D. polymorpha</i>	0.1	0.08	1.52	0.8
	1	0.93	3.84	1.74
	10	10.32	319.2	6.07

5.2.5 Statistical analysis

In accordance with Dallas et al. 2013 and Dallas et al. 2016a, relative mRNA expression ratio (RER) of key genes (*sod*, *cat*, *gst*, *hsp70* and *hsp90*) was quantified using REST (v 2009), from PCR efficiencies calculated using LinReg PCR software (version 11, (Ramakers et al. 2003; Ruijter et al. 2009)) and threshold cycle (Cq). Values were normalised to the geometric mean of Cq determined for reference genes actin (*act*) and elongation factor 1 (*ef1*), using control samples to calibrate.

All statistical analyses were carried out using the statistical software R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), <https://www.r-project.org/>). Data were checked for normality distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with visual examination of QQ-plots. The non-parametric Kruskal-Wallis test was used if assumptions were not met; comparison between treatment groups was determined using a Dunn's pairwise comparison with Bonferroni correction. Where assumptions were met, a one-way ANOVA was run with Tukey's post hoc tests. To compare between treatment groups, a Wilcoxon rank sum test with Holm-Bonferroni correction was used. Any correlation between variables was determined using a Pearson's correlation coefficient. Level of significance for all tests was set at $p < 0.05$ (*) and data presented as mean \pm standard deviation, unless otherwise stated.

5.3 Results

During the experimental (exposure) periods, no spawning or mortality of the mussels occurred in either of the species. Metal and ^{32}P concentrations, along with water quality measurements are presented in Table 5.1. Results of the ICP-MS and LSC analyses confirmed that achieved values were in line with expected concentrations across all treatments.

5.3.1 Genotoxic response following *in vivo* exposures to ^{32}P

Fig. 5.2 shows the mean (\pm S.D) % tail DNA damage, MN per 1000 cells and γ -H2AX induction in MG and DP gill cells, following exposure to varying ^{32}P concentrations for 10 days. All control (unexposed) treatments showed a low degree of damage across all biomarkers, indicative of good health in study species.

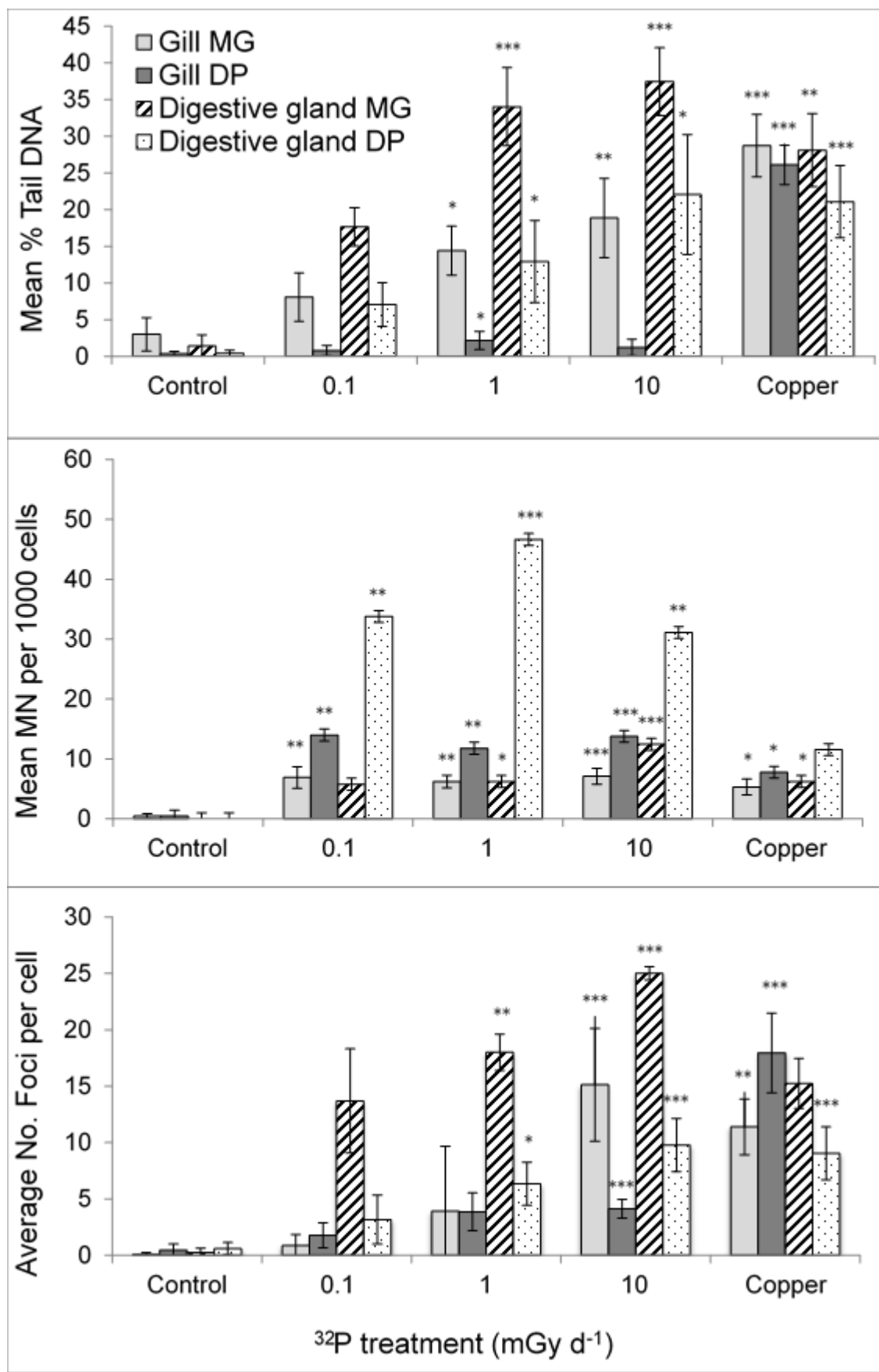


Figure 5.2. Genotoxic effects in *M. galloprovincialis* and *D. polymorpha* gill cells following a 10-day exposure to ^{32}P . Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. SD is standard deviation of mean data. $n = 9$.

5.3.1.1 Comet assay to determine DNA strand breaks

The results indicate that in terms of DNA damage (% Tail DNA, Fig. 5.2), both the tissues showed a significant dose dependant increase in response to ^{32}P exposure ($p < 0.01$), particularly in MG tissue and DP digestive gland ($p < 0.001$). Control samples showed low levels of damage at around 1-2%.

The highest level of damage was evident in MG digestive gland cells for both 1 and 10 mGy d⁻¹ doses, with around 34-37% tail. The relatively low dose rate to DP gill cells (Table 5.2) resulted in minimal damage to DNA across all treatments, with a slight increase for 1 mGy d⁻¹ dose ($p < 0.05$). MG tissues showed greater DNA damage across all ^{32}P treatments when compared to the equivalent DP tissues ($p < 0.01$). Interestingly, there was no statistical difference between DNA damage for the 1 and 10 mGy d⁻¹ doses, a trend repeated for MN and γ -H2AX formation across all tissue (apart from MG gill cells, γ -H2AX, $p < 0.05$).

5.3.1.2 Analysis of micronuclei (MN) induction

MN formation (Fig. 5.2) did not follow the dose-dependent response pattern which was evident for DNA damage (comet response) and γ -H2AX induction, while ^{32}P had a significant effect ($p < 0.001$) there was little difference between treatment groups. DP digestive gland showed significantly greater MN formation than MG digestive gland, gill and DP gill, especially for 1 mGy d⁻¹ dose at 47 MN per 1000 cells. Similarly, MN induction in DP gill cells was higher than that in MG gill, across all treatments, however not significantly so ($p = 0.92$).

5.3.1.3 Induction of γ -H2AX foci

A positive correlation (Fig. 5.3, 4) was evident between DNA damage and γ -H2AX in all but the DP gill exposure (MG digestive gland: $r = 0.91$, $p < 0.001$, MG gill: r

= 0.6, $p < 0.001$, DP digestive gland: $r = 0.79$, $p < 0.001$). The 10 mGy d⁻¹ treatment produced the greatest degree of damage across all species and tissues, in comparison to controls ($p < 0.001$). In keeping with DNA damage, the greatest number of γ -H2AX foci was in MG digestive gland cells, across all ³²P treatments with around 13-25 foci per cell. On average, the number of foci in MG digestive gland was 3-4 fold greater than in DP digestive gland cells ($p < 0.01$). Following exposure to a dose of 0.10 mGy d⁻¹, there was no significant increase in γ -H2AX or DNA damage, in either of the species or tissues.

5.3.2. Transcriptional expression of key genes

PCR efficiencies for studies genes were: *actin* (*act*): 1.79, *elongation factor 1* (*ef1*): 1.79, *catalase* (*cat*): 1.81, *glutathione-s-transferase* (*gst*): 1.81, *superoxide dismutase* (*sod*): 1.80, *heat shock protein 70* (*hsp70*): 1.75 and *heat shock protein 90* (*hsp90*): 1.83 for MG, and *act*: 1.80, *ef1*: 1.79, *cat*: 1.82, *gst*: 1.79, *sod*: 1.78 and *hsp70*: 1.81 for DP. *hsp90* data is not included for DP as the assay failed to amplify. Relative gene expression of the selected genes are presented in figure 5.5, there was limited variation across all biological tissue and species. Gene *hsp70* was significantly upregulated in DP gill following ³²P exposure ($p < 0.05$), along with *gst* in the 1 mGy d⁻¹ treatment, however to a limited degree.

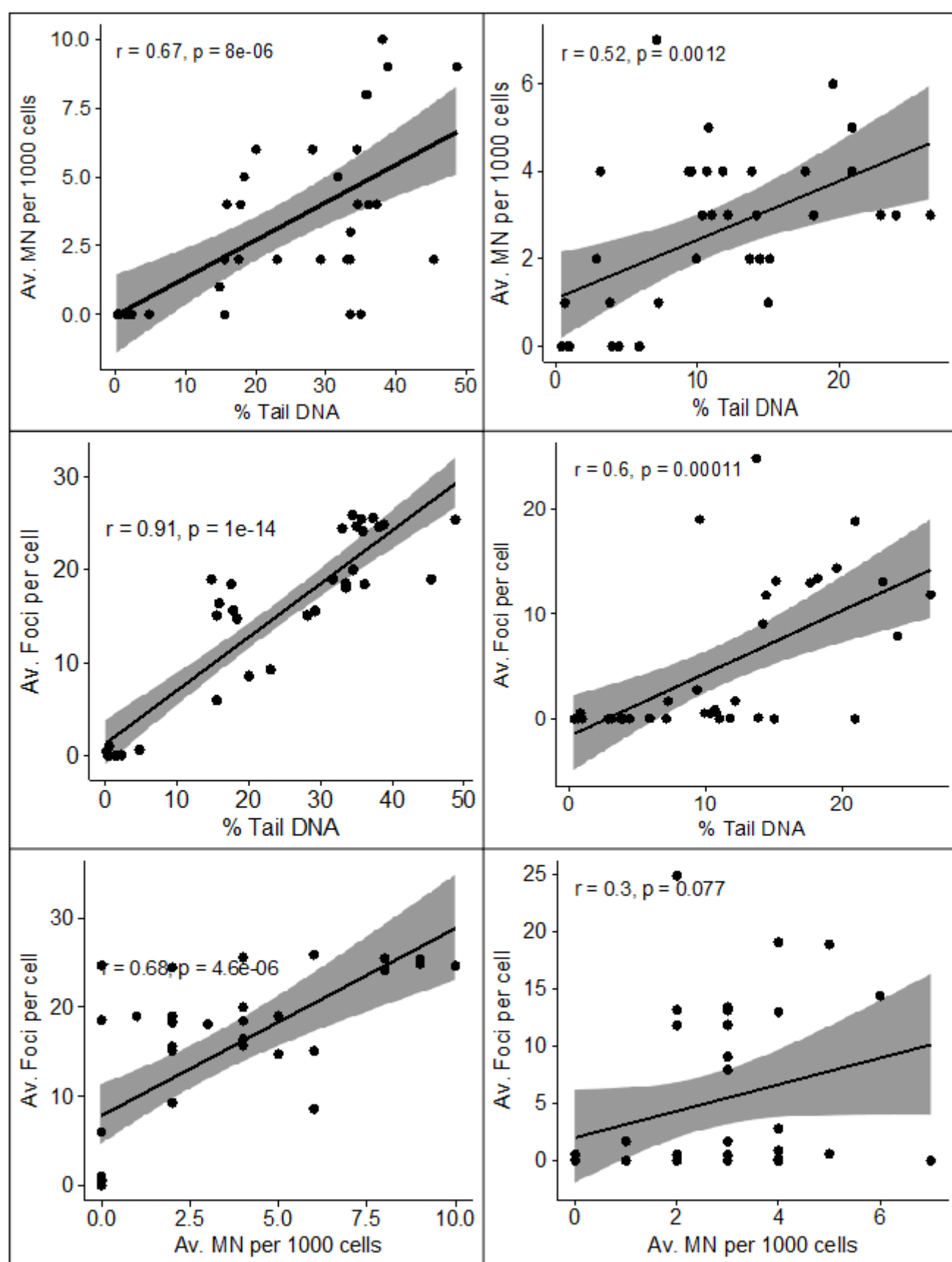


Figure 5.3. Pearson's correlation analyses of: (top to bottom) % tail DNA and induction of MN, % tail DNA and γ -H2AX foci, and induction of MN and γ -H2AX foci. *M. galloprovincialis* digestive gland (left) and *M. galloprovincialis* gill (right). $n = 9$.

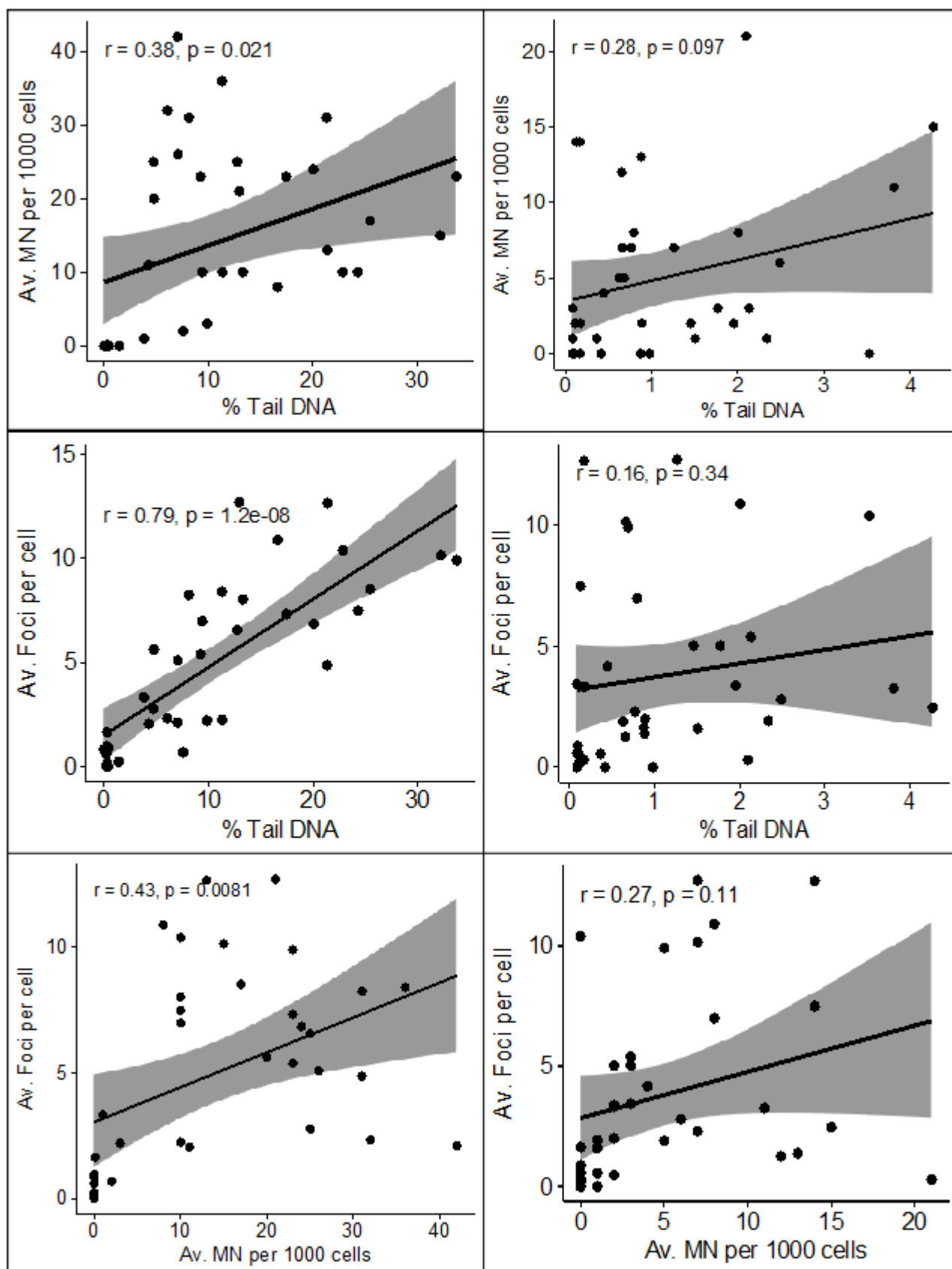


Figure 5.4. Pearson's correlation analyses of: (top to bottom) % tail DNA and induction of MN, % tail DNA and γ -H2AX foci, and induction of MN and γ -H2AX foci. *D. polymorpha* digestive gland (left) and *D. polymorpha* gill (right). $n = 9$.

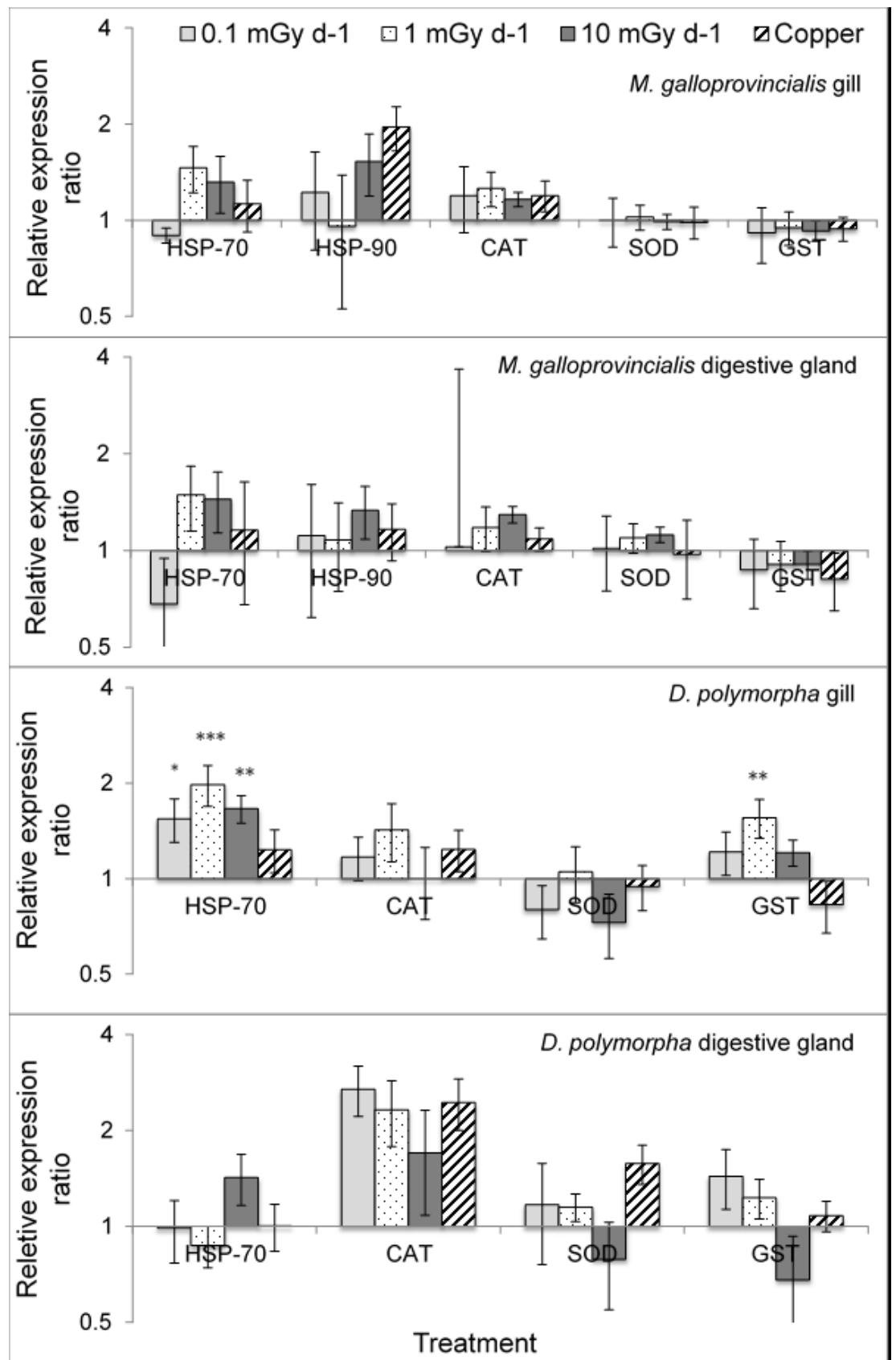


Figure 5.5. Relative expression ratios (RER) of key genes in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10-day exposure to ^{32}P . Data are normalised for reference genes (*ef1*, *actin*) and controls. Error bars indicate the 95% confidence intervals. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05, 0.01, 0.001$) from the corresponding control. $n = 9$.

5.3.3 Whole-body and tissue specific dose rates

Dose rates for whole body and specific tissues (i.e. gill and digestive gland) are illustrated in Table 5.2. Whole body dose rates were 0.10, 0.97, 10.66 for MG and 0.08, 0.93, 10.32 mGy d⁻¹ for DP, in line with predicted values. The highest dose rates were evident in digestive gland tissue, with rates around 39 (MG) and 31-fold (DP) higher than whole body dose in the 10 mGy d⁻¹ treatment. Whole body and gill tissue dose rates were comparable. A positive correlation was evident between all genotoxic biomarkers except for DNA damage in DP gill, and MN formation in both DP tissues (Fig. 5.4).

5.4 Discussion

5.4.1 Dosimetry and dose-response relationship

Our study explored the tissue specific effects following exposure to ³²P on marine and freshwater bivalves, using a multi-biomarker approach. It is important to note that in the current study, ³²P was introduced in a highly bioavailable form (i.e. radiolabelled ATP). The observed biological responses may be reflective of this form of radioactive phosphorus (Vernon et al. 2018). Our study firstly suggests that gill and digestive gland tissues are sensitive and reliable cell types for assessing IR-induced responses, this deviates from previous bivalve studies where haemocytes are predominantly chosen as target cell types to study biological responses (Jha et al. 2005; Jha et al. 2006; Jaeschke et al. 2015; Dallas et al. 2016a; Pearson et al. 2018). Secondly, our study has highlighted the necessity to evaluate response in several organs, as greater ³²P activity levels within digestive gland tissue in particular has induced a greater genotoxic response. Whole body dose rates (Table 5.2) in this instance are likely to mask the potential biological effect of ³²P. At dose rates below the screening value of

10 $\mu\text{Gy h}^{-1}$ (0.24 mGy d^{-1}) (Andersson et al. 2008; Andersson et al. 2009), whole body dose estimations (MG 0.10, DP 0.08 mGy d^{-1}) would suggest a minimal risk to individual or population. However, MG digestive gland dose as an example (4.32 mGy d^{-1}), is 43-fold greater than whole body. As noted by Jha (2008) and Jaeschke et al (2011), it is vital to determine tissue specific effects in response to a wide range of contaminants, only then are we able to implement adequate protection policies, and suitable radiation benchmarks.

5.4.2 ^{32}P induced genotoxic response in gill and digestive gland cells

5.4.2.1 Comet assay to determine DNA strand breaks

The present study revealed significant DNA damage (as % Tail DNA) in MG gill tissue and the digestive glands of both species, highlighting strong correlations between dose rate and the response. Our results are in line with previous studies where exposure to doses between 12-485 $\mu\text{Gy h}^{-1}$ (tritium, ^3H) induced significant damage in haemocytes of *Mytilus* sp. (Jha et al. 2005, 2006; Dallas et al. 2016a; Pearson et al. 2018). In both species the highest DNA damage was evident in digestive gland cells, most likely due to high ^{32}P accumulation, and therefore dose rate. To our knowledge, few radiobiological studies have investigated IR-induced response in digestive organs, despite them playing major roles in metabolism, immune defence, and as a primary sink for many aquatic contaminants (McDonald et al. 1993; Cartier et al. 2004; Dimitriadis et al. 2004; Banni et al. 2017; Faggio et al. 2018; Sforzini et al. 2018b). Mussel digestive glands accumulate and process nutrients, which are distributed to reproductive tissues during gonad development; as such, a key concern with digestive gland damage is the possible influence on reproductive success (Sastry and Blake 1971; Dimitriadis et al. 2004). Interestingly, no significant level of DNA damage is

evident at dose rates of 0.10 mGy d⁻¹ in either tissue or species, this result is in contrast to Jha et al (2006), where low doses of tritium (0.30 mGy d⁻¹, 96 h) caused a 2.8-fold increase in damage compared to control cells in haemocytes of mussels. This trend is continued in Zebrafish (*D. rerio*, 96 hpf larvae), where exposure to low dose-rates (¹³⁷Cs, 0.8 mGy d⁻¹) caused significantly increased damage (Gagnaire et al. 2015). Disparity may be down to numerous factors, including differential sensitivity between cell types or species, radionuclide LET and exposure length, or physiological factors (i.e. reproductive stage, metabolism, health status) (Nalepa et al. 1991; Jha 2008; Pearson et al. 2018). In terms of DNA damage, little change was evident in DP gill tissue, with a slight significant increase (statistically, but probably not biologically) noted for 1 mGy d⁻¹ dose. Mussel gills filter suspended particulates directly from the surrounding media to specific organs (Jørgensen 1982). They play a major role in respiratory processes, nutrient uptake and digestion (David and Fontanetti 2005; Gómez-Mendikute et al. 2005). The large surface area and close proximity to aquatic contaminants makes them a prime tissue for biomonitoring and ecotoxicological studies and their sensitivity to numerous pollutants is well documented (Mersch et al. 1996; Parolini et al. 2011b; Al-Subiai et al. 2012; Dallas et al. 2013, 2016a, 2018; Canesi et al. 2014). Interestingly, while DNA damage was relatively low in DP gill, there was significant MN induction (at around 13 MN per 1000 cells) across all ³²P treatments. As MN, a biomarker of 'effect', are apparent following cell division, any increase in its frequency can be regarded as a longer term sign of damage, in comparison to the comet and γ-H2AX assays which provide a brief snapshot in time. The lack of correlation between end points measured in DP gill may be a result of DNA-DNA or DNA protein crosslinks that inhibit tail migration during the comet assay (Hartmann et al. 2001; Klobučar et al. 2003).

As previously noted, ^{32}P incorporates directly onto the ribose-phosphate backbone of replicating DNA and isotopic decay (^{32}P to ^{32}S) breaks the initial strand (SSB), and by close proximity emitted electrons can cause DSBs, resulting in a higher proportion of strand breaks than other beta emitters (i.e. ^{90}Y , ^{131}I , (Cheng et al. 2015). The comet assay in this instance is useful in showing current, non-specific (i.e. single or DSBs) DNA damage, often regarded as more sensitive when combined with other biomarkers (Frenzilli et al. 2009). Future studies would benefit from measuring DNA damage post exposure at multiple time points, as to monitor possible DNA repair in different cell types. This would be particularly relevant for MG digestive gland cells, where a high degree of DNA damage is paralleled ($r = 0.91$, $p < 0.001$) by increased $\gamma\text{-H2AX}$ foci, suggesting the recruitment of DNA repair proteins (Kuo and Yang 2008).

5.4.2.2 Induction of $\gamma\text{-H2AX}$ foci

IR has the potential to damage cellular DNA and to maintain genomic integrity and function, repair enzymes/mechanisms are required (Hoeijmakers 2001; Sancar 2004). The long-term biological impact of ^{32}P is dependent on the efficiency of such DNA repair mechanisms, as unrepaired DNA lesions may result in genetic mutation, leading to cancer formation or cell death (cytotoxicity). As noted by Kuo and Yang (2008), H2AX is rapidly phosphorylated to form $\gamma\text{-H2AX}$, a crucial factor in DSB repair response, following irradiation with IR. $\gamma\text{-H2AX}$ induction has been noted at dose rates of 70-550 mGy d⁻¹ in Zebrafish ZF4 cells and embryos, in response to ^{137}Cs exposure (Pereira et al. 2011; Pereira et al. 2014). In Medaka (*O. latipes*) fish erythrocytes, a high frequency of $\gamma\text{-H2AX}$ foci (2 h, 39.9 ± 45.05) were noted following an acute exposure (15 Gy) to ^{137}Cs , and in line with our study, were well correlated with DNA damage (as measured by

the comet assay) (Sayed et al. 2017). The present study suggests that mussels are able to employ repair mechanisms following ^{32}P induced DNA damage, while no significant increase in $\gamma\text{-H2AX}$ formation was evident at 0.10 mGy d^{-1} , a dose dependant response was demonstrated overall. If we compare data obtained from comet and $\gamma\text{-H2AX}$ assays, several similarities are apparent, and a good correlation is evident between all end-points (all but DP gill cells). $\gamma\text{-H2AX}$ foci represent DSBs in a 1:1 manner (Kuo and Yang 2008), the strong correlation between comet and $\gamma\text{-H2AX}$ assays suggests that the majority of DNA damage evident is in the form of DSBs, as opposed to SSBs, alkali-labile sites or DNA cross-links.

$\gamma\text{-H2AX}$ is dephosphorylated rapidly after DNA repair and is therefore time-dependant. Foci peak in size at around 30 min and decrease in number over time (Sedelnikova et al. 2003; Ivashkevich et al. 2011, 2012). As noted by Sayed et al (2017), $\gamma\text{-H2AX}$ foci per cell (Medaka erythrocytes) peaked 2 h after exposure, and declined steadily over time (24 h). This study is limited however, by small, inconsistent sample numbers and very few cells counted per sample, as suggested, a minimum of 50-100 cells should be counted per sample (Redon et al. 2009; Oommen et al. 2016b). Whilst care was taken to process the tissues for analysis immediately after exposure, in our study it is highly possibly that all end-points would vary over time. Monitoring during, immediately after, and h/days post exposure would allow for a more clear indication of permanent, or repairable effects.

Differing repair capacities are evident between the digestive glands of MG and DP. In terms of MG digestive gland, a high degree of DNA damage and $\gamma\text{-H2AX}$ foci induction (~ 25 foci/cell, 10 mGy d^{-1}), combined with relatively low MN induction suggests efficient DNA repair mechanisms. In comparison, in DP

digestive gland, γ -H2AX foci induction is 2.5-4.3 fold lower than MG, but MN formation is significantly greater. This could indicate a less efficient DNA repair mechanism, or that the system was overwhelmed. It is important to note that γ -H2AX foci form at both unprogrammed and programmed DSBs. Due to low foci number in control samples (~ 0.3 foci/cell), we are confident in attributing foci formation to ^{32}P exposure in our study (Sedelnikova et al. 2003; Revet et al. 2011). To avoid misinterpretation of results, it is important that baseline levels of cellular DSBs are determined prior to exposures (or via control treatments).

5.4.2.3 Analysis of micronuclei (MN) induction

MN represent fragments of chromosomes or whole chromosome formed via misrepair of DNA DSBs (Fenech et al. 2011; Bolognesi and Fenech 2012). The capacity of IR to induce MN has previously been reported in bivalves (*M. edulis*) and fish (*Catla catla*, *Cyprinus carpio*, *Oryzias latipes*) (Jha et al. 2005, 2006; Anbumani and Mohankumar 2012; Jaeschke et al. 2015; Kumar et al. 2015; Sayed et al. 2017; Hurem et al. 2018). Our results suggest that MN formation is the most sensitive endpoint to ^{32}P , as significant MN formation was present even at the lowest dose (0.10 mGy d^{-1} , all but MG digestive gland). DP digestive gland cells had a vast number of MN in comparison to all other ^{32}P treatments at around 31-47 MN/1000 cells. This suggests that DNA damage overwhelmed the repair capacity of the cells, resulting in less reversible, more permanent effects.

A correlation is evident between DNA damage and increased MN frequency in all but DP gill (MG digestive gland: $r = 0.67$, $p < 0.001$, MG gill: $r = 0.52$, $p < 0.01$, DP digestive gland: $r = 0.38$, $p < 0.021$). This is in contrast to previous studies where strong positive correlations are noted (Bolognesi et al. 2004; Hagger et al. 2005; Jha et al. 2005; Canty et al. 2009; Kumar et al. 2015). No significant

variation was evident between ^{32}P treatments, and there is little distinction between species, cell type, or treatment, with MN frequency ranging between 6-14 MN/1000 cells (excluding DP digestive gland). While the reason for this is unclear, differing results suggest varying MoA's between beta-emitting radionuclides (tritium, ^{32}P) on the DNA in mussels. Our study clearly shows the benefit of adopting a multi-biomarker approach in measuring IR-induced genetic damage. The combination of biomarkers aids the detection of differing aspects of genotoxicity (DNA repair and response) and clastogenicity (Jha et al. 2005; Araldi et al. 2015; Kumar et al. 2015).

5.4.3 Expression of key genes

IR has the potential to damage biological molecules, in turn altering their function. Despite this there are few mechanistic studies about such processes in aquatic invertebrates (Farcy et al. 2007; Farcy et al. 2011; AlAmri et al. 2012; Devos et al. 2015; Dallas et al. 2016a). Interestingly, while genotoxic response to ^{32}P was evident in both species, there was little change in genes involved in cell stress defence mechanisms (protein folding or regulation of oxidative stress). Slight up-regulation of *hsp70* (all ^{32}P treatments, $p < 0.05$) and *gst* (1 mGy d⁻¹, $p < 0.001$) was noted in DP gill, albeit to a relatively limited degree.

IR generates ROS via the radiolysis of water (Barillet et al. 2011; Graupner et al. 2016). To minimise the detrimental effects of ROS, antioxidant enzymes such as *sod*, *cat* and *gst* are employed. It could be expected that genes involved with oxidative stress response, detoxification and/or cellular defence would be upregulated when exposed to IR. This was noted following acute exposure to ^{137}Cs in *K. marmoratus* embryos, where antioxidant enzyme-coding genes (e.g. *gst*, *cat*, *mn-sod*, *cu/zn-sod*) were significantly upregulated at dose rates of 5 Gy

(Rhee et al. 2012). However, our findings are in line with Devos et al (2015) who noted significant genotoxic response (i.e. DNA damage, as measured by the comet assay) following exposure of *C. gigas* to ^3H (0.07-1.1 mGy d⁻¹), but no change in gene expression levels (*hsp70*, *hsp90*, *hsc72*, *gst*, *mdr*, *cyp1a*, *sod*, *mt1&2* and *p53*). This was also noted in MG gill cells following exposure to tritium (7 d, ~0.36 mGy d⁻¹), where DNA damage significantly increased ($p < 0.05$) in comparison to control treatments, but gene expression levels remained unchanged (slight increase in *hsp70-1* and *rad51*, 72 h) (Dallas et al. 2016a). However, after 3 days (72 h, ~0.36 mGy d⁻¹) significant upregulation was noted in all genes (*hsp70-1*, *hsp70-2*, *hsp90*, *mt20*, *p53*, *rad51*) (Dallas et al. 2016a), suggesting time as an important factor in the transcriptional expression of genes. As suggested by Devos et al (2015), the lack of change noted at a molecular level may suggest different sensitivities for end points, or disparity in the MoA of toxicity. It is possible that evident genomic damage resulted from direct interaction of ^{32}P with DNA, either through isotopic decay or the subsequent release of high-energy beta particles, and to a lesser degree via the generation of ROS.

Our gene expression analysis included a limited number of stress response genes, as the current study was limited to readily available gene sequences in both species. Future studies would benefit from studying a wider range of genes, particularly those involved in DNA damage and repair (i.e. *p53*, *ogg1*, *rad51*), or via a more open ended approach. For example, adopting a high-throughput transcriptomic (e.g. RNAseq) or proteomic approach, which allows the measurement of expression levels in thousands of genes/proteins, could be utilised to identify early IR-induced responses, and would aid the identification of mechanisms involved in an organism's toxicity response to IR.

5.4.4 Environmental implications and future research

While ^{32}P occurs in small quantities in the environment, when accumulated into biological tissue it is able to induce significant genomic damage. The marine and freshwater species chosen for this study represent suitable models to investigate ^{32}P induced toxicity. The multi-species approach adopted here could be regarded as more robust, and realistic than single-species experiments (Chapman 2002; Solomon and Sibley 2002; Canty et al. 2009; Schnug et al. 2014). It is important to note that variations in ^{32}P speciation and therefore bioavailability between salt and freshwater may have influenced species response. We are however able to establish a genotoxic response in both MG and DP, even at relatively low ^{32}P levels. Increased genomic instability may ultimately have a detrimental effect at higher levels of biological organization, from individual to long-term population level effects (Jha 2008).

In the natural environment, ionising radionuclides are part of a complex mix of aquatic contaminants that can place combined pressure on biota. Field studies are arguably more environmentally realistic in determining the true biological effect of contaminants, taking into account the plethora of additional stressors (i.e. predation, disease, population density, food availability). However, as noted by Farcy et al (2007), the complexity of the natural environment makes it difficult to link damage response to a particular source. Future studies would benefit from: (a) combined field and laboratory studies, (b) laboratory experiments using a more realistic, flow-through exposure set-up (to note, due to health, safety, logistical and economic reasons a static exposure was utilised in this study), (c) multi-stressor and/or multi-species exposures, and (d) use of a wide range of radionuclides and exposure conditions. Knowledge of the behaviour, transfer and

biological impact of radionuclides within aquatic systems allows for the development of adequate management and protective strategies.

5.5 Conclusions

In terms of the short-lived radionuclide, ^{32}P , a greater tissue concentration is paralleled by increased dose rate, and biological damage in two anatomically similar bivalve species. In terms of DNA damage and DDR, the marine species, MG appears to be slightly more sensitive on an immediate, short-term level, possibly due to greater accumulation rates. However in terms of longer-term damage, high MN formation in DP digestive gland cells suggests a more permanent response. This low-dose, chronic study is the first to adopt a multi-species, multi-biomarker approach in investigating tissue specific ^{32}P induced biological response, along with dose-response relationships. In terms of radiation science, this approach could be readily adopted to study impact of other radionuclides either alone or in combination with other environmental stressors.

Chapter 6

Evaluation of interactive effects of phosphorus-32 and copper on marine and freshwater bivalve molluscs



In preparation:

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6.1 Introduction

Radionuclides and metals co-exist in the aquatic environment, their increased prominence resulting primarily from human activities (e.g. industrial discharge, nuclear power generation, accidents or weapons tests, mining, wastewater treatment) (Hu et al. 2010). Nuclear industries as an example co-dispose of radionuclides and non-radioactive waste. The requirement for large volumes of water for cooling processes typically results in NPPs being located close to large water bodies (i.e. oceans, lakes), where waste products are disposed. IR emitted from discharged radionuclides can enter the environment via controlled or accidental (nuclear accidents: Chernobyl, Fukushima) release, whilst discharges of contaminants are largely regulated, little is known about interactive effects.

Exposure to a mixture of stressors, which in isolation may not induce significant damage, may cause deleterious effects on organism health through additive or synergistic mechanisms when acting in combination (Mothersill et al. 2007). Alternatively, combinations may have an antagonistic effect, where the addition of one stressor may offer protection against another. As demonstrated by Tran et al (2007), selenium (Se, 4 $\mu\text{g L}^{-1}$) had a protective effect in *M. edulis* haemocytes, against exposure to known toxic agent, mercury (Hg^{2+} , 20 $\mu\text{g L}^{-1}$). To improve the basis for environmental and human risk assessments, it is crucial to advance our understanding on multi-stressor induced effects on biological, and ecological levels.

It is well accepted that radionuclides and metals can readily concentrate in the tissues of aquatic organisms, posing a threat to both humans via consumption, and biota by trophic level transfer (Carvalho 2018). When present within a cell, such contaminants have varying potential to cause significant damage to

molecules such as DNA, either directly or indirectly, the extent dependent on concentration, LET, distribution and biological half-life. IR could interact with other environmental stressors (metals, organics or physical agents such as temperature) in different ways (i.e. additively, synergistically, antagonistically) and could modify observed biological effects (Manti and D'Arco 2010).

In context, IR-induced biological response has been investigated in a wide range of aquatic biota, predominately following exposures to long-lived radionuclides such as ^{137}Cs and ^3H . Gene expression alterations, arguably the first step towards response to any contaminant have been demonstrated in molluscs (*C. gigas*, *Mytilus* spp.), arthropods (*D. magna*, *T. japonicas*), echinoderms (*P. lividus*) and rotifer (*B. koreanus*), following exposure to IR, with focus predominantly on specific cell stress marker genes, namely heat shock chaperone proteins (i.e. *hsp70*, *90*), along with markers of oxidative stress (*gst*, *cat*, *sod*) and DNA repair (*p53*, *rad51*). At low ^3H doses ($\sim 15.58 \mu\text{Gy h}^{-1}$), Dallas et al (2016a) noted upregulation of genes involved in protein folding, DNA DSB repair and cell cycle checkpoint control in MG, transcriptome level effects were well correlated with those at higher organisational levels (genetic, DNA damage). Given that IR is capable of inducing a range of responses at different levels of biological organisation, which are often determined simultaneously, adoption of a holistic and integrated approach is required to assess the induced biological responses.

In light of this, mathematical models provide the conceptual and mathematical formalism to integrate molecular, cellular and whole animal processes (Allen and McVeigh 2004; Allen and Moore 2004; Moore and Noble 2004). Previous studies have shown that Principal Component Analysis (PCA) and network complexity can be used as an indicator of homeostasis or health in cellular systems (Moore

2010; Moore et al. 2015; Sforzini et al. 2015; Sforzini et al. 2018a). Modelling is essential for the extrapolation of explanatory frameworks that facilitate the development of a predictive capacity for estimating outcomes or risk associated with stressful environmental conditions (Moore and Noble 2004; Moore 2010; Moore et al. 2015). Previous studies on mussels and earthworms have shown that there is a strong relationship between the first principal component (PC 1) for responses of numerous stress biomarkers and lysosomal membrane stability (LMS), as an indicator of cellular health (Moore et al. 2006; Sforzini et al. 2015; Sforzini et al. 2017; Sforzini et al. 2018a). Multivariate statistical analysis, including PCA was used to integrate multi-biomarker data in test organisms (MG and DP) and specific tissues.

As IR is not an isolated threat to aquatic biota, several studies have utilised laboratory exposure scenarios to determine combined toxicity of multiple environmental stressors (Olsvik et al. 2010; Heier et al. 2013; Dallas et al. 2016a). Olsvik et al (2010) and Heier et al (2013) investigated interactions between radionuclides (^{60}Co) and environmentally relevant metal concentrations (Cu, Al, Cd) in Atlantic salmon (*S. salar*). The addition of Al and Cd appeared to reduce the impact of gamma-irradiation by modifying transcriptional induction of oxidative stress-responsive genes (including *p53*, glutathione reductase, glutathione peroxidase, metallothionein) (Olsvik et al. 2010). This trend was not continued following exposure to Al and Cu, suggesting varying MoA's in toxicity response. Potential IR-metal induced synergistic, antagonistic or additive effects are yet to be fully explored in aquatic invertebrates.

In context, short-lived radionuclide ^{32}P was utilised as a cost-effective, accessible surrogate for more environmentally prominent beta and gamma emitting radionuclides (e.g. ^{137}Cs , ^{90}Sr). As previously demonstrated (Vernon et al. 2018),

^{32}P readily accumulated in the tissues of mussels, particularly digestive gland (see chapter 4). Detrimental ^{32}P -induced responses (DNA damage, MN and γ -H2AX foci induction) have been noted at dose rates as low as 0.1 mGy d^{-1} , in both digestive gland and gill cells (see chapter 5).

Metals such as copper (Cu) can be highly toxic to organisms at concentrations present within marine and freshwater environments. Cu-induced deleterious effects in aquatic invertebrates are well studied (Al-Subiai et al. 2011; Vosloo et al. 2012; Brooks et al. 2015; Xu et al. 2016), and much research has focused on interactions between Cu and additional stressors (metals, ocean acidification, temperature, pesticides, microplastics) (Clayton et al. 2000; Bouskill et al. 2006; Trevisan et al. 2011; Maria et al. 2013; Lewis et al. 2016). However, to our knowledge no studies have determined the interaction between IR and Cu in mussels.

In this work, the effects of ^{32}P and Cu, alone and in combination were studied in two ecologically relevant adult bivalve species, MG and DP. Mussels are excellent models for investigating the relevance of low metal/IR doses, they are well established in ecotoxicological research and as such, a large number of validated biological effect endpoints are available to be measured and quantified in a range of cell types (i.e. haemocytes, digestive gland and gills cells). A suite of biomarkers, from molecular to behavioural levels were measured in the digestive gland and gill cells of two mussel species, following exposure to a range of Cu and ^{32}P concentrations/doses, alone and in combination. ^{32}P dose rates of 0.10 and 1 mGy d^{-1} were reflective of a generic (all species) “no effect” screening dose rate of $10 \text{ }\mu\text{Gy h}^{-1}$ (0.24 mGy d^{-1}), the chosen Cu concentration ($18 \text{ }\mu\text{g L}^{-1}$) was in line with environmentally realistic values and adopted from previous

validation studies described in chapter 3 (Andersson et al. 2008; Andersson et al. 2009). The overall aims and objectives of this study were (a) to adopt an integrated, multi-biomarker approach in investigating the combined effects of Cu and ^{32}P in two bivalve species, (b) to determine relative sensitivity of marine and freshwater adult bivalves, (c) to determine potential correlations between different parameters (molecular, genetic and behavioural) studied and (d) to determine relative sensitivity between different cell types (i.e. gill and digestive gland cells). With regards to species variation, we hypothesised firstly that little disparity in response will be evident. Secondly, Cu would have an additive effect on the ^{32}P -induced responses in mussels.

6.2 Materials and methods

6.2.1 Chemicals and suppliers

Radiolabelled-ATP (Adenosine triphosphate, γ - ^{32}P), was obtained from Perkin Elmer (PerkinElmer, UK) in batches of 9.25 MBq (specific activity: 370 MBq mL⁻¹), and diluted with DI water to form appropriate working solutions, as in Vernon et al (2018). All working solutions were decay adjusted throughout the exposure.

6.2.2 Mussel exposure conditions

Adult MG and DP were collected and maintained in accordance to section 2.2. Ten-day exposures of both the mussel species were performed between September-October 2017, and were staggered by two weeks for ease of analysis and logistical reasons. Following collection and after a 2-week acclimation, MG and DP individuals (total weight, 35 g L⁻¹) per labelled beaker were exposed to the following exposure scenarios in triplicate: (a) 0.1 mGy d⁻¹, (b) 0.1 mGy d⁻¹ + Cu, (c) 1 mGy d⁻¹ and (d) 1 mGy d⁻¹ + Cu. The Cu (CuSO₄·5H₂O, 99% purity)

concentration used in combination with ^{32}P was $18\ \mu\text{g L}^{-1}$. Control and positive control (Cu, $56\ \mu\text{g L}^{-1}$) treatments were run alongside. ^{32}P activity levels in water were calculated in a previous study (section 2.8; Vernon et al. 2018), to meet expected nominal dose rates, ^{32}P activity concentrations for $0.1\ \text{mGy d}^{-1}$ were $993\ \text{Bq L}^{-1}$ (MG) and $579\ \text{Bq L}^{-1}$ (DP), and for $1\ \text{mGy d}^{-1}$, $9930\ \text{Bq L}^{-1}$ (MG) and $5786\ \text{Bq L}^{-1}$ (DP). Activity concentrations in water were calculated from preliminary experiments (data not included). The selection of lower and higher concentrations (positive control) of Cu were based on previous studies (Chapter 3).

Mussels were fed 2 h prior to water changes (50 %), on days 3, 5, 7 and 9, as described in earlier studies (Vernon et al. 2018). Water quality parameters were measured routinely, before and after water changes. Phosphorus-32 and Cu activity levels were determined using water samples ($1\ \text{mL}$, in duplicate), taken ~30 minutes after each water change and processed for ICP-MS or liquid scintillation counting (LSC), as in section 2.7.2 and 2.8.3.2. Data from LSC counting and ICP-MS analysis confirmed that achieved values were in line with expected concentrations across all treatments.

6.2.3 Sampling procedures

After exposures, gill and digestive gland tissue was dissected from each individual and stored as followed until use: $\frac{2}{3}$ tissue stored in tube on ice until cell isolation, $\frac{1}{3}$ stored in RNeasy lysis buffer (1.5 mL, Fisher UK) at $-20\ ^\circ\text{C}$. All other soft tissue and shell was discarded.

6.2.4 Biological assays

6.2.4.1 Isolation of digestive gland and gill cells

The procedure to obtain gill and digestive gland cells for genotoxic assays was adopted from previous studies (Vincent-Hubert et al. 2011), and outlined in section 2.3.2. Supernatant was then used in subsequent assays, providing cell viability, checked using the Trypan Blue exclusion dye assay (section 2.4) (Strober2001), was <90% across all treatments (data not shown).

6.2.4.2 Comet assay to determine DNA strand breaks

The comet assay was performed using gill and digestive gland cell suspension (150 μ L), as described in section 2.5.2.

6.2.4.3 Analysis of micronuclei (MN) induction

Gill and digestive gland cell suspension was adhered and fixed as described in section 2.5.1, before staining with 20 μ L ethidium bromide (20 μ L of 20 mg L⁻¹). Cells ($n = 500$) were scored per slide and results are reported as mean MN per 1000 cells, in keeping with other data from our research group (Dallas et al. 2013).

6.2.4.4 Induction of γ -H2AX foci

γ -H2AX foci were determined in gill and digestive gland cells, following procedures outlined in 2.5.3. All slides, including procedural blanks were coded and scored at random, and 50 cells were counted per individual/slide.

6.2.4.5 Determination of transcriptional expression of key genes

Gill and digestive gland tissue was dissected immediately after exposure and stored in RNAlater at -80 °C (R0901, Sigma-Aldrich Company Ltd UK) until use.

RNA extraction, cDNA synthesis and qPCR were performed in accordance to sections 2.5.4.1 – 2.5.4.3.

6.2.5 Behavioural observations: Valve movement and byssus attachment

As noted in section 2.6.2, valve movement or activity (i.e. whether the individual was actively filtering or had a fully closed shell) was assessed by eye, three times daily during the course of the exposure (Rajagopal et al. 2003; Hartmann et al. 2015). Byssus attachment (i.e. whether the individual is attached to either the glass beaker/other individuals) was assessed every alternate day of exposure by eye, as an indicator of mussel health (Angarano et al. 2009; Ericson et al. 2010; Martinović et al. 2016).

6.2.6 Water quality measurements and ^{32}P and Cu analyses

6.2.6.1 Determination of ^{32}P in water samples using liquid scintillation counting

All samples were collected, processed and analysed as in section 2.8.3.1. Activity concentrations were background corrected by blank subtracting from each sample, the blank was nonspiked fresh or seawater. In accordance with Jaeschke and Bradshaw (2013), CPM values that fell below the blank were assigned an activity of 0.000. All samples were decay corrected.

6.2.6.2 Determination of Cu concentration in water samples using Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Cu concentrations in water samples were determined following procedures outlined in section 2.7.2.

6.2.7 Dosimetry and the ERICA TOOL

The Tier 2 assessment module of the ERICA tool was used for dose estimation. ^{32}P was chosen as one of the ERICA tool's default isotopes (Brown et al. 2008). Tissue specific dose rate (e.g. ^{32}P dose to digestive gland) was determined by taking mean measurements during sampling (i.e. mass, height, width, length), and developing custom geometry parameters on the ERICA tool (Table 2.4), the ERICA tool was utilised in accordance to section 2.8.4. Dose rates for whole-body, gill and digestive gland tissue ^{32}P concentrations are presented in Table 6.1.

Table 6.1. Table to show (a) the expected dose rates in mGy d^{-1} and (b) the average dose rate achieved in *M. galloprovincialis* and *D. polymorpha* whole-body, digestive gland and gill tissue (mGy d^{-1}).

	Expected dose rate	Av. Dose rate (mGy d^{-1})		
		Whole body	Digestive gland	Gill
<i>M. galloprovincialis</i>	0.1	0.11	4.34	0.09
	1	0.96	38.76	0.66
<i>D. polymorpha</i>	0.1	0.08	1.53	0.8
	1	0.87	3.72	1.68

6.2.8 Statistical analysis

In accordance with Dallas et al (2013) and Dallas et al (2016a), relative mRNA expression ratio (RER) of genes was quantified using REST (v 2009), from PCR efficiencies calculated using LinReg PCR software (version 11, Ramakers et al. 2003; Ruijter et al. 2009) and threshold cycle (Cq). Values were normalised to the geometric mean of Cq, determined for reference genes actin (*act*) and elongation factor 1 (*ef1*), using control values to calibrate.

Statistical analyses were operated using the statistical software R (RStudio, R 3.4.3 GUI 1.70 El Capitan build (7463), <https://www.r-project.org/>). Where applicable, data was checked for normality distribution (Shapiro-Wilk test) and homogeneity of variances (Levene's test), with visual examination of QQ-plots. The non-parametric Kruskal-Wallis test was used if assumptions were not met; comparison between groups (i.e. specific tissue or species) was determined using a Dunn's pairwise comparison with Bonferroni correction. Where assumptions were met, a one-way ANOVA was run with Tukey's post hoc tests. Comparison between groups was determined using a Wilcoxon rank sum test with Holm-Bonferroni correction. Level of significance for all tests was set at $p < 0.05$ (*) and data presented as mean \pm standard deviation, unless otherwise stated.

6.2.8.1 Multivariate analysis

Biomarker data for gill and digestive gland tissue in both species were analysed using non-parametric multivariate analysis software, PRIMER v 6.1.5 (PRIMER- ϵ Ltd., U. Auckland, New Zealand; Clarke 1999; Clarke & Warwick. 2001). All data were log transformed [$\log_n(1+x)$] and standardised to the same scale. Correlations between biomarkers were tested using scatter plot matrices; while

PCA, hierarchical cluster analysis and non-metric multi-dimensional scaling analysis (MDS, plots not shown), derived from Euclidean distance similarity matrices, were used to visualize dissimilarities between sample groups. The results were tested for significant differences between treatments using analysis of similarity (PRIMER v6 - ANOSIM), which is an approximate analogue of the univariate ANOVA, and reflects on differences between treatment groups in contrast to differences among replicates within samples (the R statistic). Under the null hypothesis H_0 ("no difference between samples"), $R = 0$ and this was tested by a non-parametric permutations approach; there should be little or no effect on the average R value if the labels identifying which replicates belong to which samples are randomly rearranged. Behavioural responses were excluded from the PCA due to non-paired samples.

The PRIMER v6 - BIO-ENV routine (Spearman's Rank Correlations) linking multivariate biomarker response patterns was used to identify potential "influential biomarkers", small subsets of biomarkers capturing the full PCA biomarker response pattern.

6.3 Results

No mortality or spawning occurred in either mussel species throughout the duration of the experiment. Cu and ^{32}P concentration, along with water quality measurements are presented in Table 6.2. Whole body and specific tissue (i.e. gill and digestive gland) dose rates are illustrated in Table 6.1. Whole body dose rates were 0.11 and 0.96 for MG and 0.08, 0.87 mGy d⁻¹ for DP, in line with predicted values.

Table 6.2. Water quality parameters (pH, temperature, salinity and dissolved oxygen [DO]), copper concentrations in water ($\mu\text{g L}^{-1}$) and ^{32}P concentrations in water (Bq L^{-1}), for both species. Data is presented as mean \pm standard deviation.

Water parameters	<i>M. galloprovincialis</i>	<i>D. polymorpha</i>
pH	8.1 \pm 0.06	8.1 \pm 0.08
Temp ($^{\circ}\text{C}$)	14.6 \pm 0.24	14.7 \pm 0.23
Salinity	36.7 \pm 3.90	0.3 \pm 0.03
DO (%)	99.4 \pm 2.15	93.2 \pm 1.33
Copper water conc. ($\mu\text{g L}^{-1}$)		
Control	1.9 \pm 0.08	0.3 \pm 0.02
0.1 mGy d $^{-1}$ + Cu	14.0 \pm 0.72	17.1 \pm 0.21
0.1 mGy d $^{-1}$	2.4 \pm 0.24	0.3 \pm 0.07
1 mGy d $^{-1}$ + Cu	15.8 \pm 0.49	17 \pm 0.14
1 mGy d $^{-1}$	4.1 \pm 0.56	0.4 \pm 0.16
56 $\mu\text{g L}^{-1}$	40.9 \pm 2.09	56.0 \pm 0.60
^{32}P water conc. (Bq L^{-1})		
Control	0.8 \pm 1.04	0.2 \pm 0.58
0.1 mGy d $^{-1}$ - 993 / 579	1246.3 \pm 360.94	573.2 \pm 444.04
1 mGy d $^{-1}$ - 9930 / 5786	9712.8 \pm 1235.16	4641.0 \pm 2128.19

6.3.1 Genotoxic response following *in vivo* exposures to ^{32}P and Cu

Fig. 6.1 shows mean (\pm S.D) (A) % tail DNA damage, (B) MN per 1000 cells and (C) γ -H2AX induction in MG and DP gill and digestive gland cells, following exposure to varying concentrations of ^{32}P and Cu, alone and in combination for 10 days. Potential correlation within the biomarker data are displayed on Figs. 6.2a and b, A – D. Control (unexposed) treatments show a low degree of damage across all biomarkers, indicative of good health in both mussel species.

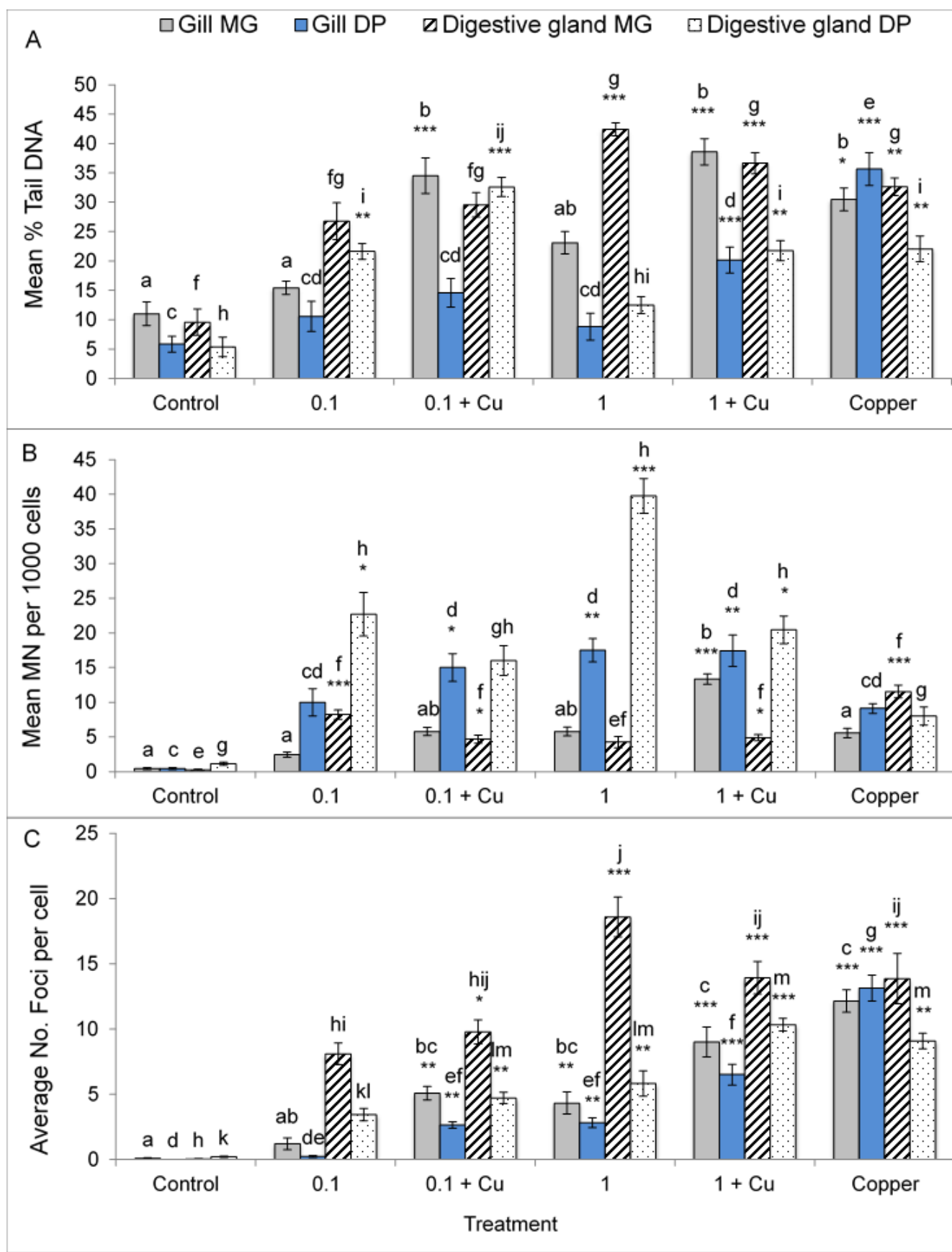


Figure 6.1. Genotoxic effects and subsequent repair in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10 day exposure to ^{32}P and Cu, alone and in combination. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. Letters are indicative of significant differences ($p < 0.05$) between species tissue (i.e. MG gill tissue). SD is standard deviation of mean data. $n = 9$.

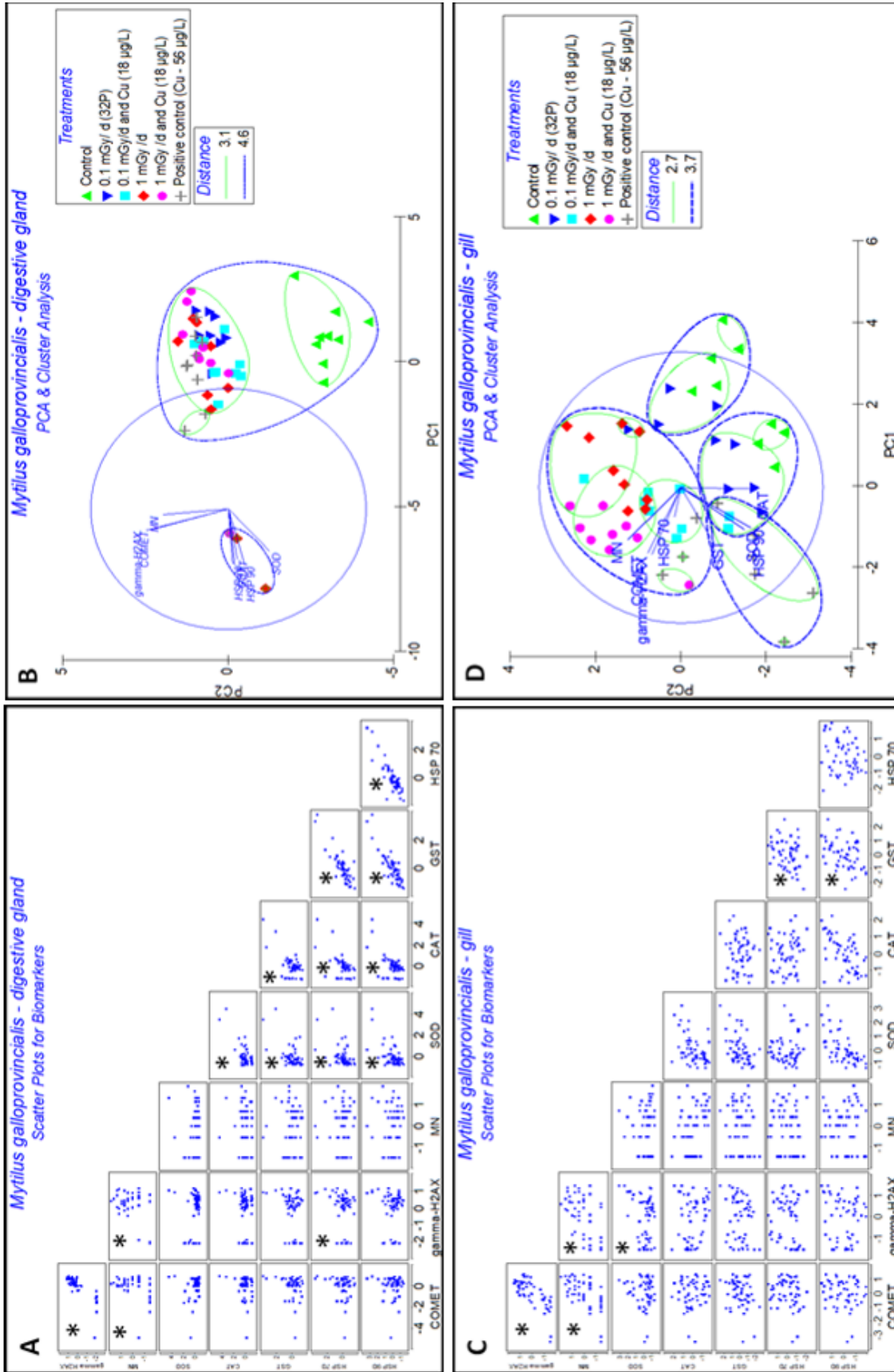


Figure 6.2a. Scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data, not including behavioural response. Vectors indicate the directionality of specific biomarkers. (A, B) *M. galloprovincialis* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$)

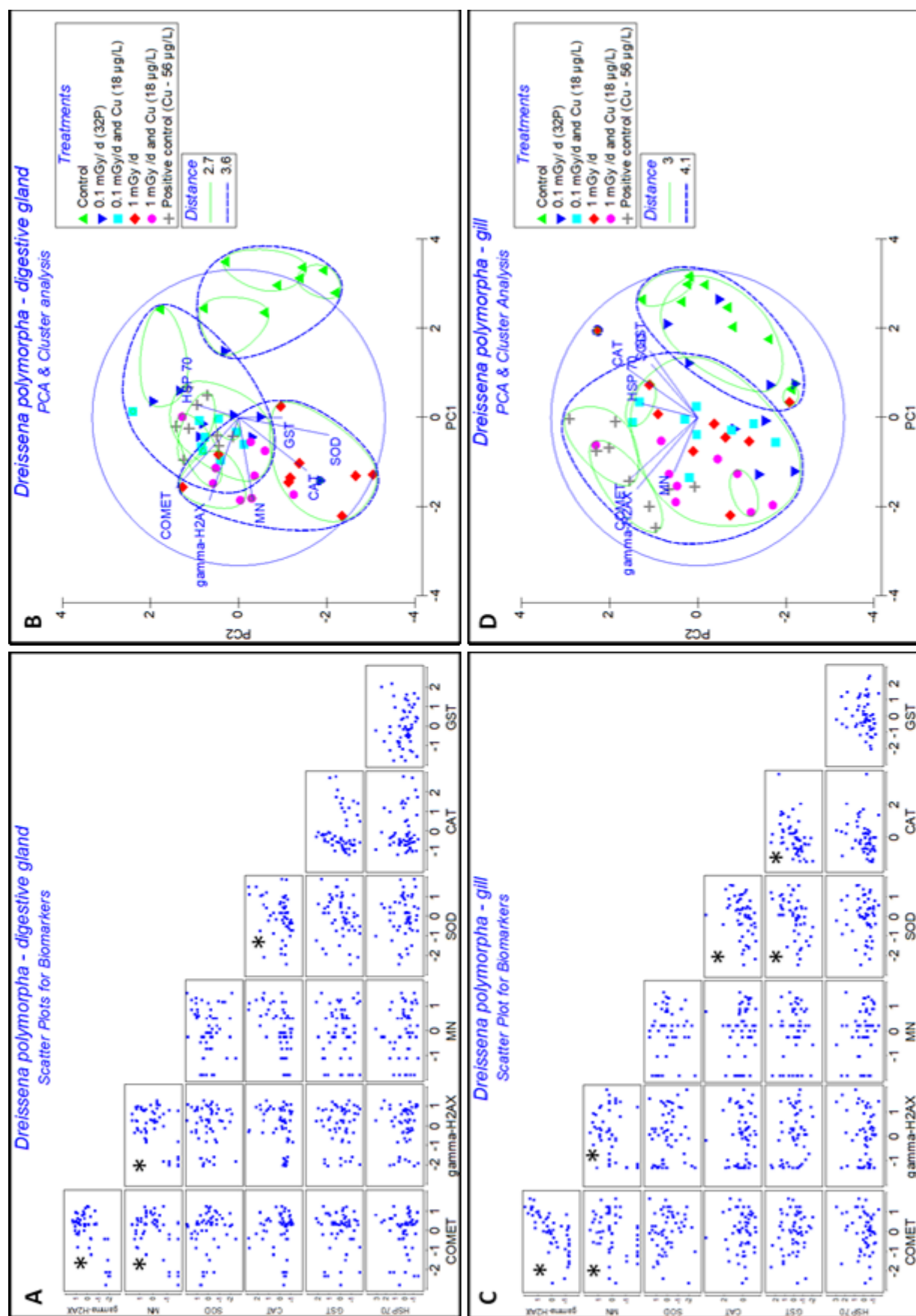


Figure 6.2b. Scatter plots (A, C), principal component (PCA) and cluster analysis of the biomarker data, not including behavioural response. Vectors indicate the directionality of specific biomarkers. (A, B) *D. polymorpha* digestive gland and (C, D) gill tissue. Asterisks (*) are indicative of significant differences ($p < 0.05$)

6.3.1.1 Comet assay to determine DNA strand breaks

While the only significant ($p < 0.01$) interaction between stressors was evident in MG gill, between the 0.1 and 0.1 + Cu treatments, Cu had a weak interaction with ^{32}P across all treatments excluding MG digestive gland (1 and 1 + Cu), where the addition of Cu increased the damaging effect of ^{32}P (Fig. 6.1A). The lowest degree of damage was evident in DP gill tissue across all treatments, % Tail DNA was only significantly greater than controls with the addition of Cu (1 + Cu, $p < 0.001$). This trend was continued in MG gill (both treatments). Cu appeared to have the greatest interaction with ^{32}P within this tissue, where % Tail DNA was increased by 2.3- and 1.7-fold (0.1 and 1, respectively).

6.3.1.2 Analysis of micronuclei (MN) formation

Trends noted in DNA damage response were not continued in MN formation (Fig. 6.1B), as Cu did not appear to interact with ^{32}P in a detrimental manner (excluding MG gill). No significant difference is noted between treatments in DP gill, MG and DP digestive gland. The greatest degree of damage was evident in DP digestive gland, particularly in the 1 mGy d⁻¹ treatment at 40 MN/1000 cells. Interestingly, Cu appeared to have an antagonistic interaction with ^{32}P , where MN induction decreased when exposed to both stressors. In terms of relative sensitivity, DP shows a higher MN frequency in gill and digestive gland cells over its marine counterpart, across all treatments (excluding negative/positive controls). However only significantly so between gill cells in the 1 mGy d⁻¹ treatment ($p < 0.05$).

6.3.1.3 Induction of γ -H2AX foci

Fig 6.1C shows the average number of γ -H2AX foci per cell, indicative of DSBs. In keeping with DNA damage, a large number of γ -H2AX foci were evident in MG digestive gland, particularly in 1 mGy d⁻¹ treatment at 19 foci/cell. On average, γ -H2AX foci in MG digestive gland was 2.3– to 3.1-fold greater than DP. In relation to the corresponding control, no significant increase in γ -H2AX foci/cell were noted at the lowest ³²P treatment of 0.1 mGy d⁻¹ across both species and tissues, however, foci number significantly increased with the addition of Cu (MG digestive gland: $p < 0.05$, MG and DP gill, DP digestive gland: $p < 0.01$).

6.3.2 Transcriptional expression of key genes

PCR efficiencies for studies genes were: *actin* (*act*): 1.79, *elongation factor 1* (*ef1*): 1.79, *catalase* (*cat*): 1.81, *glutathione-s-transferase* (*gst*): 1.81, *superoxide dismutase* (*sod*): 1.80, *heat shock protein 70* (*hsp70*): 1.75 and *heat shock protein 90* (*hsp90*): 1.83 for MG, and *act*: 1.80, *ef1*: 1.79, *cat*: 1.82, *gst*: 1.79, *sod*: 1.78 and *hsp70*: 1.81 for DP (primer details, table 2.1). *hsp90* data is not included for DP as the assay failed to amplify. Relative gene expression of the selected genes are presented in figure 6.3. Overall there was limited variation across all biological tissue and species, with no change evident in MG digestive gland. At 1 mGy d⁻¹, *cat* was downregulated in MG gill, but upregulated in DP digestive gland ($p < 0.001$). Downregulation of *cat* was further noted in MG gill 1 + Cu treatment, but to a lesser extent ($p < 0.01$). In response to Cu, *sod* was significantly upregulated in MG gill ($p < 0.01$).

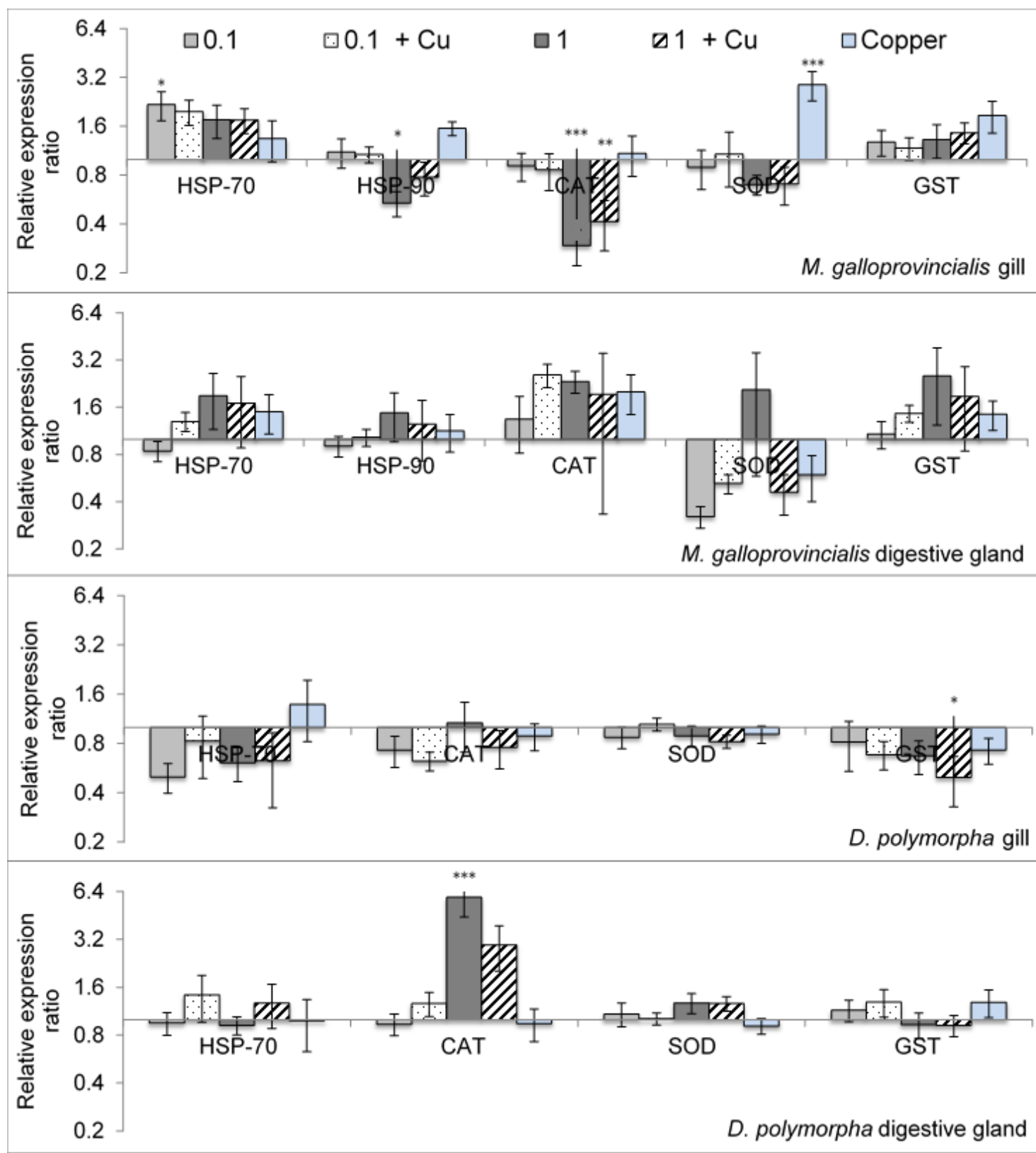


Figure 6.3. Relative expression ratios (RER) of key genes in *M. galloprovincialis* and *D. polymorpha* gill and digestive gland cells following a 10-day exposure to ^{32}P and Cu, alone and in combination. Data are normalised for reference genes (ef1, actin) and controls. Error bars indicate the 95% confidence intervals. Asterisks (*, ** or ***) are indicative of significant differences ($p < 0.05$, 0.01, 0.001) from the corresponding control. $n = 9$.

6.3.3 Multivariate analysis of biomarker reactions

Analysis of the potential correlations within the biomarker data indicated that most of the biological parameters were not strongly correlated (Fig. 6.2a and 2b, A & C), with several exceptions (primarily comet [measure of DNA damage], γ -H2AX and MN assays, across all tissues, $p < 0.05$). PCA and MDS (MDS plots not shown) combined with cluster analysis showed that the experimental treatments were clearly distinct from the control groups (Fig. 6.2a and b, B & D). The percentage of variance explained by PC1 and PC2 were as follows: DP digestive gland, 32.6% and 21.4%; DP gill, 33.2% and 24.3%; MG digestive gland, 47.5% and 27.1% and MG gill, 32.9% and 28.4%. The ANOSIM analysis gave global significances of $p < 0.001$ for both tissues and species. In DP gill tissue, pairwise analysis showed that all treatments were significantly different from one another ($p < 0.05$), digestive gland tissue of this species showed similarly distinct differences between pairs of treatments except for 0.1 mGy d^{-1} v $0.1 \text{ mGy d}^{-1} + \text{Cu}$. MG gill tissue showed distinct differences between pairs of treatments except for 0.1 mGy d^{-1} v 1 mGy d^{-1} , and 1 mGy d^{-1} v $1 \text{ mGy d}^{-1} + \text{Cu}$. However, the digestive gland tissue of MG showed greater overlap in treatments, with no significant differences between 0.1 mGy d^{-1} v $1 \text{ mGy d}^{-1} + \text{Cu}$, 0.1 mGy d^{-1} v positive Cu Control, 1 mGy d^{-1} v $1 \text{ mGy d}^{-1} + \text{Cu}$, & $1 \text{ mGy d}^{-1} + \text{Cu}$ v positive Cu control. The PCA results for this tissue showed that the experimental treatments were more strongly grouped together than in any other tissues (Fig. 6.2B).

As already stated, most biomarkers (particularly the expression of key genes) were not correlated with each other, and the BIO-ENV routine for various combinations of biomarkers indicated that there were no influential biomarkers among the various combinations capable of capturing the full PCA biomarker response pattern. Principal component (PCA) coupled with hierarchical cluster

analysis and ANOSIM results for all treatments showed that both ^{32}P and Cu had a detrimental effect on the genetic integrity and oxidative stress status in the four tissues tested (Figs. 6.2a and b, A - D).

6.3.4 Behavioural observations: Valve movement and byssus attachment

No significant variation was found between experimental treatments in terms of active filtering (valve movement) or byssus attachment (Fig. 6.4), a possible result of small n numbers. Control mussels showed increased attachment with time, overall MG showed a greater propensity to attach than DP, with all 3 attached from exposure day 3 (Fig. 6.4a). Valve activity was variable throughout the exposure, with MG generally showing more active filtration, independent of treatment (Fig. 6.4b).

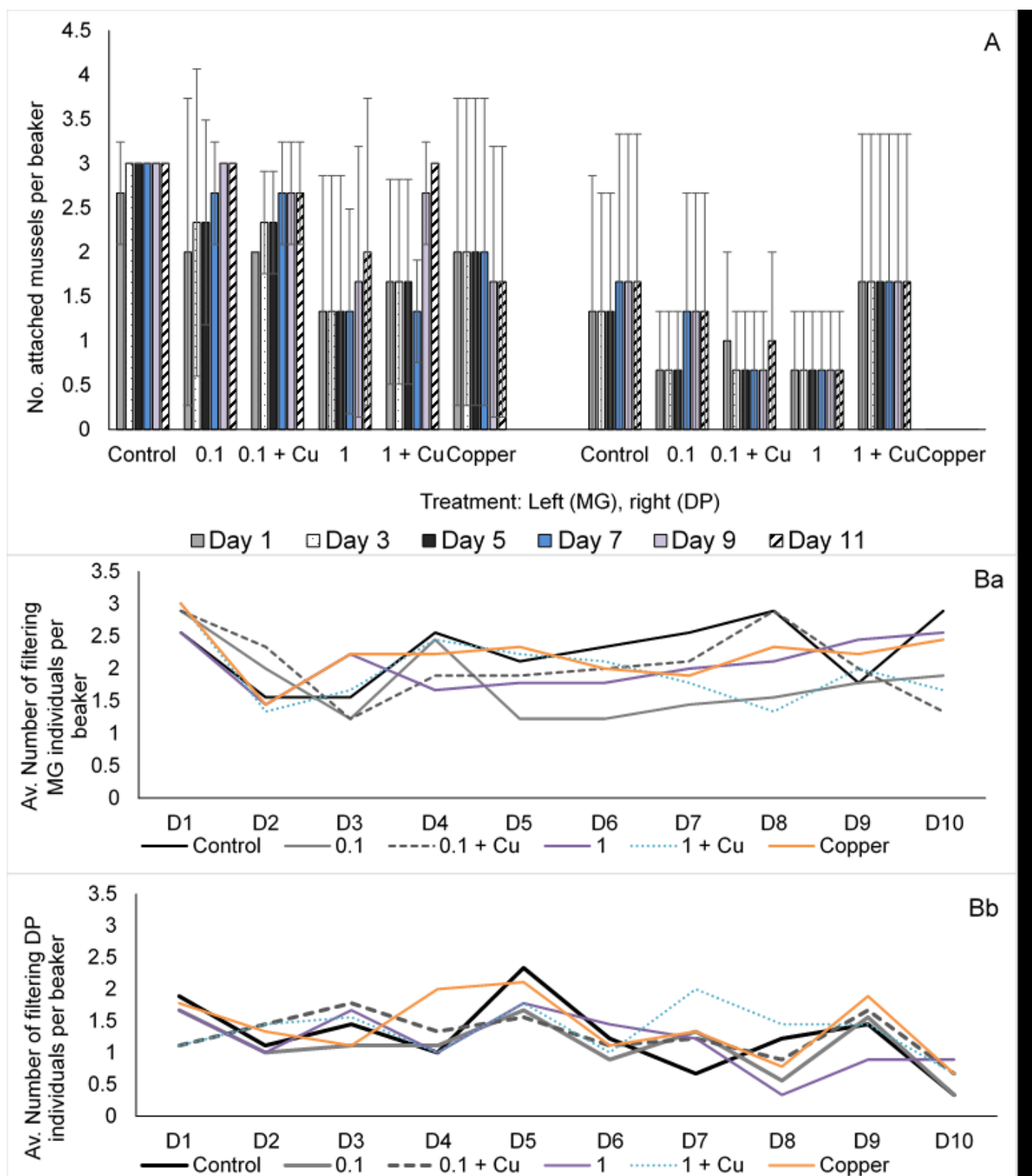


Figure 6.4. Behavioural effects in *M. galloprovincialis* (MG) and *D. polymorpha* (DP) following a 10 day exposure to ^{32}P and Cu, alone and in combination. (A) Number of attached mussels per beaker, on days 1, 3, 5, 7, 9 and 11 of exposure, (Ba, b) Average number of mussels (Ba: MG, Bb: DP) actively filtering each experimental day, per beaker. SD is standard deviation of mean data. $n = 3$.

6.4 Discussion

This novel study is the first to explore the interactive effects of IR and Cu on two aquatic bivalve species using a tissue specific, multi-biomarker approach. Qualities of radionuclides and speciation of metals (i.e. physical and chemical properties) in aquatic systems has a large influence on bioavailability and subsequent toxicity (Gunten and Beneš 1995; Richards et al. 2011). It is well documented that Cu, focusing on the toxic ionic form (Cu^{2+}), forms complexes with natural organic matter, decreasing bioavailability and at lower salinities (i.e. freshwater) becoming more abundant (Grosell et al. 2007). Despite this, little is known on the possible interactions between Cu and lesser studied radionuclides such as ^{32}P , and the combined influence of water parameters [i.e. pH, salinity, dissolved organic carbon (DOC), alkalinity]. Due to time and logistical constraints, certain parameters (e.g., DOC) were not determined during this experiment. It is possible that differing water chemistry (i.e. salt and freshwater), along with chemical interactions between stressors could have affected the noted biological effects in each species. However, correlation between accumulations of Cu and ^{32}P (in isolation) in soft tissues with increasing genotoxicity in cells (gill and digestive gland, Chapters 3, 4) were evident, in each species independently.

6.4.1 Biomarker interactions

Recent developments spanning multiple fields are leading to the discovery of prognostic biomarkers that may be suitable for use as risk indicators of biological damage (Moore et al. 2006; Jenkins et al. 2011; Ortiz et al. 2011; Berghella et al. 2014). It is probable that many biomarkers only exhibit a response in a part of the “health status space” (Depledge et al. 1993; Moore et al. 2004; Moore et al. 2006), where they indicate whether a reaction has taken place and may even indicate

health status within a narrow range, or what has induced the response, but they do not generally indicate health status of the animal for the whole range from healthy to irreversible damage (Köhler et al. 2002). In terms of environmental prognostics, the first step is to relate biomarker responses to the health status of individual organisms, by mapping said responses against an integrated “health status” indicator (Köhler et al. 2002; Allen and Moore 2004; Moore et al. 2004; Moore et al. 2006). PCA is an effective method for integrating biomarker data (i.e. DNA damage, MN formation, gene expression etc.) into a “health status space”, reducing the multi-dimensionality of the problem to a simple two dimensional representation (Chatfield and Collins 1980; Allen and Moore 2004). PCA is commonly used as a cluster analysis tool and effectively captures the variability in a dataset in terms of principle components. PCA has facilitated modelling the integrated responses of multiple biomarkers in the context of “health status space” (Allen and Moore 2004; Moore et al. 2006). PCA and associated statistical tests have previously shown that lysosomal biomarkers (as indicators of health status), in combination with the comet assay provide an effective integrated assessment of the adverse effects on physiological function and genetic integrity (genotoxicity) (Sforzini et al. 2015; Sforzini et al. 2017; Dallas et al. 2018; Sforzini et al. 2018a). In continuation with earlier studies, current findings demonstrate that PCA can aid interpretation of multiple biomarker responses and pathological reactions to multiple environmental stressors (i.e. IR and metals).

6.4.2 ^{32}P induced genotoxic response in gill and digestive gland cells

The majority of IR-induced toxicity studies have focused on single radionuclide exposures. DNA damage as a biomarker has been noted at dose rates ranging from 0.8-41666 $\mu\text{Gy h}^{-1}$ in a wide range of biota, including marine bivalves

(*Mytilus* spp., *C. gigas*, *P. perna*, *C. fluminea*, *P. malabarica* & *M. casta*) (Jha et al. 2005, 2006; Godoy et al. 2008; Farcy et al. 2011; AlAmri et al. 2012; Kumar et al. 2014). PCA and MDS, combined with cluster analysis demonstrate a clear distinction between controls and experimental groups, across both species, tissue and biomarker (Fig. 6.2a and b, B & D). In keeping with previous work (see Chapter 5, section 5.3.1.1), no significant change in DNA damage was noted at lower ^{32}P doses of 0.10 mGy d^{-1} (Excluding DP digestive gland, present study). Interestingly, where previous findings demonstrated significantly increased % Tail DNA in both species and cell types at 1 mGy d^{-1} , only MG digestive gland was significantly increased. The present study had greater baseline levels of DNA damage of ~5-11%, compared to <5% in previous literature, this along with individual differences may account for such variation.

Of interest, exposure of the mussels to ^{32}P alone caused no significant increase in % Tail DNA (Excluding DP [0.1] and MG digestive gland [1 mGy d^{-1}]), but when combined with Cu, % Tail DNA was statistically greater than controls in MG gill and DP digestive gland at 0.10 mGy d^{-1} , and all species and tissues at 1 mGy d^{-1} . Cu in isolation, at environmentally relevant concentrations ($18 \mu\text{g L}^{-1}$) was found to have no significant effect on % Tail DNA in MG or DP gill cells (see Chapter 3, section 3.3.2.2), when in combination with ^{32}P there appears to be an additive effect on mussels. Whilst not significantly so, the addition of Cu to IR exposures also increased $\gamma\text{-H2AX}$ foci induction, across both species, tissue and dose rate (Excluding MG digestive gland). This apparent additive effect of Cu on the genotoxicity of ^{32}P on marine and freshwater mussels is the first reported. Cu-contaminant induced effects have been noted in previous literature. In *M. edulis*, Cu-induced ($0.1 \mu\text{M}$) damage to DNA and lipids was noted as significantly greater under low pH conditions (reflective of ocean acidification), in comparison to

controls (Lewis et al. 2016). Similarly, combined Cu ($10 \mu\text{g L}^{-1}$) and IR (^{60}Co , 70 mGy) exposure was found to induce significantly depleted glutathione compared to exposure to Cu alone, in presmolt *S. salar* (Heier et al. 2013).

Aquatic biota are continuously exposed to both endogenously and environmentally generated contaminants, giving cause for the development of highly effective biochemical mechanisms that afford the ability to protect and defend on multiple biological levels. H2AX is quickly phosphorylated to form γ -H2AX, a crucial factor in DSB repair response and therefore a relevant, useful technique in radiation science, where the MoA of ^{32}P in particular is mediated by induction of DNA DSBs (Kuo and Yang 2008; Cheng et al. 2015). Pereira et al (2011), Urushihara et al (2012), Pereira et al (2014) and Sayed et al (2017) have successfully utilised this technique to assess DDR in fish (*O. latipes* and *D. rerio*) following acute exposures to ^{137}Cs , but to our knowledge, is yet to be utilised in mussels.

The γ -H2AX assay is further validated when utilised alongside classical DNA damage techniques, such as comet and MN assays, relationships have been demonstrated between such biomarkers (Pereira et al. 2011; Sayed et al. 2017). In our study, γ -H2AX was strongly correlated with DNA damage across all tissues ($p < 0.05$), correlation was not noted between other molecular or genetic biomarkers (Fig. 6.2a and 2b, A, C). The greatest induction of γ -H2AX foci was noted in MG digestive gland, specifically at 1 mGy d^{-1} (~ 19 foci/cell), followed by the $1 + \text{Cu}$, positive Cu control, $0.1 + \text{Cu}$ and 0.1 mGy d^{-1} treatments. At significantly higher dose rates of 10 mGy d^{-1} (^{137}Cs), less than 5 foci were present per zebrafish ZF4 cell (Pereira et al. 2011). As noted in section 5.4.2.2, γ -H2AX foci occurs rapidly post-irradiation (30 min – 2 h) and decrease in number over

time, as both studies measured damage immediately (~10-40 min) post exposure, varying radiosensitivity may be a result of species, radionuclide source or associated MoA (Sedelnikova et al. 2003; Ivashkevich et al. 2011,2012). Trends in γ -H2AX foci induction in MG digestive gland followed DNA damage, but not MN formation, where MN/1000 cells remained uniform respective of experimental treatment. The presence of MN is generally regarded as more permanent damage, suggesting that the induced DSBs were easily repairable. This was further noted in MG gill.

In keeping with previous studies (Chapter 5) a greater degree of MN formation is noted in DP gill and digestive gland cells, relative to associated MG tissue across all treatments, suggesting a significant impact on DNA integrity. Unexpectedly, Cu has a weak antagonistic interaction with ^{32}P in terms of MN induction, where MN/1000 cells decrease in DP digestive gland (both ^{32}P concentrations), although not significantly so. It is possible that Cu-specific repair mechanisms, as a by-product buffer against the more permanent effects induced by IR as an isolated threat.

As described in previous literature (Vernon et al. 2018), ^{32}P concentrates differentially in gill and digestive gland tissue, with the latter accumulating 87 % (MG) and 44% (DP) of ^{32}P (proportion of whole body ^{32}P concentration), at 10 mGy d⁻¹. Similarly, Cu uptake varies between tissue, and between species, (Chapter 3). In turn, there are clear differences in tissue specific dose rate (Table 6.1), and subsequent genotoxic response to IR and metals, highlighting the importance of a multiple-tissue approach in ecotoxicological studies. Overall, the addition of Cu arguably has the lowest impact on ^{32}P -induced damage in MG digestive gland, where MG gill tissue shows far less variation between treatments

(excluding % Tail DNA, 0.1 and 0.1 + Cu). In keeping with previous findings, in terms of longer-term damage, high MN frequency in DP cells suggests a more permanent response in freshwater mussels, in relation to MG (Chapter 5).

6.4.3 Transcriptional expression of key genes

In our study, transcriptional levels of key genes showed little variation between differing treatments, such findings are in agreement with earlier studies described in Chapter 5 (section 5.4.3). Excluding significant upregulation of *sod* in MG gill, the Cu positive control (56 $\mu\text{g L}^{-1}$) caused no variation in gene expression. This in contrast to work by Xu et al (2018), where exposure to much lower Cu concentrations (2 and 8 $\mu\text{g L}^{-1}$) significantly induced the expressions of stress genes (*hsp70*, *hsp90*, *mt-10*) in *Mytilus coruscus* (haemocytes), particularly 12 d post exposure. Furthermore, significantly altered transcriptional profiles in fish (*D. rerio* and *S. salar*) have been noted following IR exposure (^{137}Cs or ^{60}Co), common MoAs associated with low-dose gamma radiation included the induction of oxidative stress and DNA damage genes (Jaafar et al. 2013; Freeman et al. 2014; Song et al. 2014, 2016). As suggested by Devos et al (2015), and highlighted in chapter 5, lack of change evident at molecular levels may be suggestive of different sensitivities for end points, or disparity in the MoA of toxicity. It is possible that evident genomic damage resulted from direct interaction of ^{32}P with DNA, through isotopic decay or release of high-energy beta particles, and to a lesser degree via ROS generation.

Due to both logistical constraints and lack of readily available gene sequences in MG and DP, we were only able to assess five key genes in this study. As noted in Chapter 5 (section 5.4.3), this provides limited information pertaining to molecular mechanistic responses to IR and Cu. Furthermore, while identification

of transcriptome variation is undoubtedly important and increasingly popular within radiobiological research, limited information can be acquired by studying genes in isolation. In recent years, there has been a movement from measuring gene expression levels to proteins, as they more accurately represent the functional molecules within a cell. It is frequently highlighted that mRNA is the first step in a long sequence resulting in protein synthesis. Being transmitters of genetic information the analysis of mRNA is not a direct reflection of the protein content within a cell, for this reason many studies have found poor correlations between the expression levels of mRNA and protein (Maier et al. 2009). Following exposure to comparable Cu concentrations ($10 \mu\text{g L}^{-1}$), Maria et al (2013) noted altered proteins associated with oxidative stress (glutathione-S-transferase) and digestion, growth and remodelling processes (chitin synthase) in MG gill tissue. Findings were confirmed in MG gill and digestive gland (Gomes et al. 2014a). Interestingly in MG gill tissue exposed to isolated Cu and benzo(a)pyrene ($10 \mu\text{g L}^{-1}$, BaP), concentrations produced higher protein alterations than when in combination, suggesting an antagonistic interaction (Maria et al. 2013). Proteomics research in relation to aquatic biota is limited by a lack of available annotated genomes and proteomes for most aquatic organisms (Slattery et al. 2012), despite this, it is a clear direction for aquatic/ecotoxicological and radiobiological research. Identifying known, and potentially novel molecular targets (genes and proteins) involved, as well as activated signalling pathways in a range of biological systems under multi-stress exposure scenarios may be the first step in predicting potential larger scale impacts on more environmentally relevant levels (Maria et al. 2013; Gomes et al. 2014a).

6.4.4 Behavioural observations: Valve movement and byssus attachment

To determine interactive effects of IR and metals on mussel health, we adopted an integrated, multi-disciplinary approach. Whilst molecular and cellular biomarkers are predominantly used in ecotoxicological studies, it is difficult to extrapolate results to higher, arguably more relevant levels of biological organisation (i.e. population, ecosystem). To aid the development of effective policies for environmental protection, it is important to understand the relationship between biomarker responses at multiple organisational levels (Moore et al. 2004).

In our study no relationship was evident between exposure and behavioural observations, nor was there a correlation with molecular and genotoxic biomarkers. Bivalve molluscs adhere to their surrounding environment via byssus threads, strong proteinaceous fibres produced by the byssal gland on the base of the foot. This allows for habituation on the hydrodynamically variable intertidal zone (Smeathers and Vincent 1979; Rajagopal et al. 2005). Byssal attachment can be utilised as an indicator of vitality, as byssal thread formation requires large amounts of energy (Zardi et al. 2007). Reduced byssal attachment in *Mytilus* spp. has been documented following exposure to antifouling agents (tributyltin chloride), ocean acidification, hypoxia and pharmaceuticals (Ericson et al. 2010; Sui et al. 2015; Martinović et al. 2016), and in DP exposed to cannabinoids and organic compounds (BHA and TBHQ) (Cope et al. 1997; Angarano et al. 2009). In contrast, byssus attachment remained fairly uniform following exposure to IR and Cu, lack of notable variation is a possible result of the relatively short exposure duration. Under more chronic regimes energy may be redirected towards protective processes and away from byssal production.

In terms of valve movement, there was little change over the duration of the exposure, irrespective of species. In the natural environment bivalves hold shell valves open to facilitate necessary biological function (i.e. respiration and feeding), valve closure can occur as a protective strategy under periods of physiological stress (Kramer et al. 1989; Rajagopal et al. 2003; Redmond et al. 2017). Avoidance behaviour (fully closed shell) was not evident in this study, suggesting (a) an inability to detect contaminants in surrounding media, or (b) lack of sensitivity at particular behavioural levels. It is important to note that behavioural endpoints are susceptible to individual variation, a larger study size may better reflect stress response (Redmond et al. 2017).

6.4.5 Environmental implications and future research

Multivariate analysis is the first stage in developing numerical and network models for environmental impact on the health of sentinel animals such as marine and freshwater mussels (Allen and Moore 2004; Moore 2010; Sforzini et al. 2015, 2017, 2018a). PCA is an effective method for integrating biomarker data into a “health status space” reducing the multi-dimensionality of the problem to a simple two dimensional representation (Chatfield and Collins 1980; Allen and Moore 2004), however, PCA and cluster analysis do not integrate the various biomarkers in a functionally meaningful way. The subsets of biomarkers used in this study do not comprehensively support the development of a broad cellular physiological network that is suited to providing a measure of “health status”, however future studies could build on the current data sets in order to develop such models in the future.

This study is certainly useful in establishing relationships between stressor and response, PCA coupled with hierarchical cluster analysis and ANOSIM results for

all treatments showed that both ^{32}P and Cu had deleterious effects on genetic integrity and oxidative stress status in species and tissues tested (Fig. 6.2a and b, A - D). However the controlled nature of laboratory experiments are not fully reflective of field conditions. A continuing challenge for scientists is to understand firstly, the complexity of contaminant interactions in differing environments, and secondly, how multiple interactive stressors affect biota at all levels of biological organisation, from molecular to ecosystem levels.

6.5 Conclusions

In conclusion, the results of this integrated, multi-biomarker study represent the most extensive data to date, obtained on the combined effects of IR and metals (Cu) in two environmentally relevant bivalve species. The results indicate that (a) genotoxic response was reflective of exposure, where Cu had an overall additive effect on ^{32}P -induced damage across several (but not all) species, cell types and dose rates, (b) freshwater mussels were susceptible to longer lasting damage, marine mussels to more immediate effects, (c) selected genes were generally unaltered in terms of transcriptional response to contaminants, independent of species and (d) mussels were not responsive to IR and Cu, alone or in combination at behavioural levels. Whilst it is difficult to extrapolate such findings to exposures in realistic environmental conditions, these data contribute to the limited information on the possible mechanisms involved in multi-stressor (IR and metals) induced response, and subsequently highlight the importance of investigating the interactive effects of pollutants on ecologically relevant mussel species.

Chapter 7

General discussion and future perspectives



7.1 Environmental radiation and dosimetry

Radiobiological protection measures have adapted to become more inclusive of both human and environmental protection (Brechignac and Howard 2001; Copplestone et al. 2004; Brechignac and Doi 2009; Bréchignac et al. 2016). The main objective of radiological research is to understand potential effects of radionuclides on the organisms inhabiting the natural environment. The overall aim being to provide the necessary scientific background for the protection for both human and non-human biota (Bréchignac et al. 2016). Radiation research, however, as with other environmental protection approaches is underpinned with uncertainty (Brechignac and Doi 2009). Current issues in assessing the ecological risks of radionuclides include (a) extrapolation of laboratory data to field conditions, (b) chronic vs acute exposure, (c) external vs internal exposure, (d) single vs multi-contaminant interactions, (e) differential radiosensitivity between species and/or life stage, (f) importance of effects on different biological levels (i.e. molecular, individual, ecosystem) and (g) accurate, adequate dosimetry (Bréchignac 2003; Brechignac et al. 2012; Dallas et al. 2012; Mothersill et al. 2018). The studies presented in this thesis aimed to address some, but not all of the critical issues related to radiological protection, with focus specifically on aquatic mussels as ecologically important species representing two different habitats.

7.1.1 Radiation dosimetry

To achieve environmental protection, accurate dosimetry and the provision of safe IR dose limits is paramount (Stark et al. 2017). In the past, application or adoption of 'Umbrella' endpoints including mortality, morbidity, reproductive success and mutations was considered to be important for environmental

protection (Brechignac and Howard 2001). It is however being realised that for low level, chronic exposure conditions such as those adopted in this study, molecular and cellular responses, in line with human and mammalian studies could be more sensitive for an environmental protection point of view. In this context, the majority (~ 67%, Fig. 1.1) of studies elucidating risks of IR at molecular and genetic levels in aquatic biota, focus on acute, external exposure to gamma emitters (^{60}Co and ^{137}Cs). To add to the existing information and to more accurately reflect environmental values, chronic, low-dose exposure regimes were adopted in this thesis, reflective of a 'no effect' screening value of $10\ \mu\text{Gy h}^{-1}$ ($0.24\ \text{mGy d}^{-1}$). At dose rates below suggested protective values ($0.1\ \text{mGy d}^{-1}$), ^{32}P induced a significant biological response, particularly in MN formation in DP gill, and the digestive gland of both species (Chapter 5). This by no means suggests that current dose limits are incorrect, or that they should be lowered, but it does highlight the need to investigate sensitivity in a broad range of species, and across multiple tissues.

Accurate dosimetry for aquatic biota is complex, with dose rate and subsequent biological response dependant on factors including internal (intake via food or water) or external exposure, CR, bioaccumulation, RBE, exposure length (chronic or acute), organism characteristics and other biotic and abiotic influences (i.e. predation, reproductive stage, water quality) (Stark et al. 2017). Biological responses induced by ^{32}P were the product of external (surrounding media) and internal dose, dose rates were calculated using the ERICA tool, in whole-body and specific tissues. As highlighted in chapter 4, differential sensitivity between biological tissues (e.g. digestive gland and gill) could result in detrimental biological responses at levels presumed to be acceptable when adopting a 'whole-body' approach.

In agreement with Dallas et al (2016b), the ERICA tool was suitable for estimating radiation dose in ecotoxicological/radioecological studies on mussels. The approach however is not without limitations. While the tool provides coverage for many organism groups (Brown et al. 2016), including RAPS, we would recommend the creation of custom geometry for the chosen study organism, where discrepancies in geometry could result in inaccurate dosimetry calculations. As an example, the default weight of marine bivalve molluscs on the ERICA tool (as of Sept 2018) is 16.5 g, where the average weight of MG (in this study) is $\sim 11.7 \pm 6.6$ g. The use of custom geometry can be further applied to determine tissue specific dose rate, to our knowledge this work is unique in adopting the ERICA tool for this purpose, in terms of marine and freshwater bivalves. While the determination of tissue specific accumulation (Bq kg^{-1}) per individual is lengthy and time-consuming, as highlighted in this study, dose rate can vary significantly between organs/tissue.

7.2 Laboratory vs. field exposures

A limiting factor of this thesis is the exclusion of field data, where due to time constraints, all exposures were conducted under controlled laboratory settings. Field studies arguably provide more ecologically meaningful data (Brechignac and Doi 2009). The inclusion of environmental variation could be considered a more representative approach, particularly as the fate of radionuclides may vary under differing environmental conditions. On the other hand, it is difficult to extrapolate the direct cause of damage under field conditions and as illustrated by Farcy et al (2007), it is important to be aware of confounding factors within the aquatic environment that may mask or heighten the detrimental impact of radionuclide exposure (e.g. IR-contaminant interaction). Specific to radiation

studies, there is an added degree of complexity in the limited number of contaminated sites (e.g. AREVA reprocessing plant of La Hague, France, Sellafield, UK, Fukushima, Japan and Chernobyl, Ukraine) available for assessment. With such few contaminated sites comes a limited number of species available as bioindicators. To our knowledge, just 6 studies have utilised natural environments with increased background IR levels in determining genetic or molecular response in aquatic biota (Tsytsugina and Polikarpov 2003; Florou et al. 2004; Farcy et al. 2007; Godoy et al. 2008; AlAmri et al. 2012; Gudkov et al. 2016a). Bivalve species adopted in this study are present in contaminated environments, such as the Chernobyl cooling ponds (DP) and coastal areas surrounding La Hague (MG) (Fetisov et al. 1992; Fiévet et al. 2006; Murphy et al. 2011). Future studies may benefit from a combined, integrative approach where investigations in the field are underpinned by experimental, controlled laboratory experiments, such as those in this study.

Laboratory-based, mechanistic studies are a useful tool in establishing a clear link between dose and effect under standardised, controlled, reproducible conditions. While it is often difficult to extrapolate data from a laboratory setting and relate to field conditions, a conservative approach is to integrate acute laboratory exposures, chronic environmental experiments and modelling studies (Bréchignac et al. 2016). To more accurately reflect real-world conditions, laboratory exposures can adopt practices including (a) flow-through exposure set-ups, (b) environmentally realistic dose rates, (c) multi-species and/or multi-stressor exposures, potentially spanning several trophic levels, (d) chronic exposures and (e) realistic maintenance regimes (i.e. feeding, temperature). Both laboratory and field studies are valuable to the advancement of radiobiological research.

7.3 Mussels as bioindicators of environmental health

7.3.1 *M. galloprovincialis* and *D. polymorpha*

A main aim of this thesis was to address potential differences in species sensitivity, as even genetically related species have been found to differ when exposed to the same stressor (Saavedra et al. 2004; Wang 2010; Brooks et al. 2015). In addition, it is well accepted that amongst aquatic biota, and life stage, there are substantial variations in radiosensitivity (Sazykina and Kryshev 2006; Sazykina 2018). In previous literature, a clear disparity is evident between marine and freshwater study species, where 72 % of invertebrate species are from a marine environment, and 86 % of fish species are fresh water (Table. 1.2). In terms of environmental radiation research, marine bivalves (*Mytilus* spp., *P. Perna*, *C. gigas*) have been used extensively, also as biological indicators of ecosystem health (Hagger et al. 2005; Jha et al. 2005; Jha et al. 2006; AlAmri et al. 2012; Dallas et al. 2016a, 2016b; Pearson et al. 2018). In contrast, in terms of IR-induced cellular response this is the first study to determine response in freshwater bivalves.

The benefits of using mussels in toxicity studies have been made clear throughout this thesis, and in other literature (Mersch and Beauvais 1997; Zhou et al. 2008; Dallas et al. 2012; Binelli et al. 2015; Beyer et al. 2017), DP were deemed suitable as inland representatives of *Mytilus* spp. In terms of relative sensitivity, a similar mechanism of action for the induction of genotoxicity between species was noted following exposure to Cu (chapter 3). While similar ³²P accumulation patterns were noted between species (chapter 4, MG > DP, total uptake), in terms of DNA damage and DDR (i.e. comet assay and gamma H2AX assay, biomarkers of exposures), the marine species (MG) appeared

slightly more sensitive on an immediate, short-term level, possibly due to greater accumulation (chapter 5). However in terms of longer-term damage, high MN formation (a biomarker of effects) in DP digestive gland cells suggests a more permanent response. Such patterns were evident with the addition of Cu (chapter 6). For a more robust, environmentally realistic exposure scenario, we recommend the adoption of a multi-species approach in future studies. As highlighted in Dallas et al (2012), there are many phyla (e.g. *porifera*, *cnidara*, *Platyhelminthes* and marine chordates) where there is limited to no studies outlining relative sensitivity to IR at cellular levels (i.e. molecular/genetic). A multi-species approach would contribute to the limited data available on the environmental impacts of IR.

Despite evident advantages of adopting mussels in ecotoxicological/radiation studies there are also limitations, primarily the tolerant nature of both MG and DP. It is suggested that studies should either concentrate on more sensitive species, or use a range of biota of varying sensitivities in combination. Furthermore, a drawback to using bivalves (particularly MG and DP) is the lack of sequenced genome. Animal models such as Zebrafish (*D. rerio*), the nematode worm (*Caenorhabditis elegans*), or the fruit fly (*Drosophila melanogaster*) are widely used as relatively inexpensive organisms with a high level of genomic sequence homology to humans, and fully sequenced genomes (Davis et al. 2014). Small fish models in particular, such as *O. latipes* and *D. rerio* offer fast maturation, allowing for use in short-term, early life stage and transgenerational toxicity tests (Koyama et al. 2008), in addition low husbandry costs, and ease of maintenance are beneficial in terms of experimental design.

7.3.2 Life history stage, transgenerational effects and epigenetics

A limitation of this study is the exclusive use of adult organisms. Biological characteristics such as organism gender, life and reproductive stage and exposure history (i.e. transgenerational inheritance) can influence sensitivity, or resistance to IR exposure. Early life stages of fish and aquatic invertebrates are often reported as more sensitive to environmental toxicants (Jha 2004), despite this they are unrepresented in scientific literature, with just one study focusing on cellular effects of ^3H on *M. edulis* embryo-larvae (Hagger et al. 2005). To fully understand the impact IR on aquatic biota and future generations, it is vital that all life stages are considered.

Environmental contaminant exposures may not only induce immediate organismal responses, but can be observed over subsequent generations in organisms whose tissues were not directly exposed to the stressor (Bhandari et al. 2015). IR-induced transgenerational effects, i.e. effects seen over multiple generations are poorly understood, yet they have the potential for broad ecological impacts. In recent years, the freshwater flea (*D. magna*), marine copepods (e.g. *Paracyclopina nana* and *Tigriopus japonicas*) and small fish species (*D. rerio*, *K. marmoratus*) have been adopted as study species due to a short life span and fast reproductive rate allowing for transgenerational research. Parisot et al (2015) used the water flea to determine survival, growth, reproduction and DNA alterations in successive generations (F0, F1 and F2) following exposure to low dose gamma-emitters (^{137}Cs , 0.007 to 35.4 mGy h⁻¹). The study found an accumulation and transmittance of DNA alterations across three generations, in parallel to an increase in sensitivity of organisms. In common with human and mammalian studies (Dubrova et al. 2000; Mughal et al. 2012; Grygoryev et al. 2013; Vandeghechuchte and Janssen 2014),

transgenerational studies are undoubtedly an important future direction for radiobiological research and protection. Understanding potential vulnerabilities of future generations will allow the implementation of appropriate radiation protection measures in the present.

A rapidly expanding field within toxicological research is epigenetics (Kovalchuk and Baulch 2008; Vandegehuchte and Janssen 2011, 2014; Merrifield and Kovalchuk 2013; Mirbahai and Chipman 2014); heritable molecular variations caused by mechanisms other than DNA sequence alteration (Jaenisch and Bird, 2003). The potential of epigenetics in human medicine, such as cancer research and immune system effects has been increasingly observed in scientific literature over the last decade (Weinhold 2006). In terms of radiation biology, epigenetic mechanisms such as DNA methylation, histone modifications and small RNAs have been studied in relation to IR-induced longer term biological effects in human and mammalian systems (Aypar et al. 2011a,b; Ilnytskyy and Kovalchuk 2011; Merrifield and Kovalchuk 2013). However, to our knowledge just one study has outlined epigenetic variation in aquatic organisms, Gombeau et al (2016) demonstrated gender and tissue specific epigenetic changes (DNA methylation) in *D. rerio* exposed to depleted uranium (2 and 20 mg L⁻¹). Considering the increase in epigenetic studies in human health and ecotoxicological research, it seems likely that over the next few years the importance of understanding epigenetic mechanisms within the field of radiobiology will become evident.

In terms of aquatic organisms, the freshwater flea (*D. magna*) is highlighted as an ideal model organism for epigenetic research, due to its rapid life-cycle, ease of culture and low cost (Connon et al. 2012). The water flea has previously been applied in radiobiological research to determine survival, growth, reproduction and DNA alterations in successive generations following exposure to γ -emitters

(Alonzo et al. 2008; Parisot et al. 2015). Epigenetic variations can in some instances be transferred to subsequent generations, even when these generations are no longer exposed to the external stressor which induced the original epigenetic modification (Vandeghehuchte and Janssen 2011). This could have serious implications for future populations. For effective environmental protection, research is required to determine the potential phenotypic and population level impact of epigenetic modification in a range of aquatic organisms, and to evaluate the persistence of radionuclide exposure-induced epigenetic response in multiple subsequent generations.

7.4 Suitable biomarkers and radiation science

While the biological mechanisms behind IR-induced damage are being increasingly explored, particularly in human and mammalian models, there are still significant gaps in our understanding. Furthermore, a main uncertainty in radiological protection is determining what should be protected, individual organisms, populations or ecosystems (Coppelstone et al. 2004). In recent years, an integrated, ecosystem approach to radiological protection has been recommended, where the environment is protected as a whole (Brechignac and Doi 2009; Brechignac et al. 2012; Mothersill et al. 2018).

While not the focus of this thesis, it would be of benefit to establish relationships between molecular and genetic level effects caused by IR, to higher levels of biological organisation (i.e. individual, reproductive, population levels). Such responses have been well established in marine and freshwater mussels in response to a range of contaminants (Bacchetta and Mantecca 2009; Potet et al. 2016; Beyer et al. 2017). This would provide a thorough, environmentally relevant

understanding of IR-induced damage on aquatic biota, on both a short- and long-term basis.

7.4.1 Advancements in radiological research: The development of novel biomarkers

IR is widely exploited in human nuclear medicine, where radioactive decay of specific radionuclides is used to target cancerous tumours (Sofou 2008; Cheng et al. 2015; Gudkov et al. 2016b). Advancements in human-based molecular techniques, such as the ‘omics’ approaches (e.g. transcriptomics, proteomics, ecotoxicogenomics) are frequently crossed over to toxicological research, they allow for the identification of novel biomarkers related to IR response (Thybaud et al. 2007; Viant 2007; Tomanek 2014).

Techniques such as RNA-sequencing (RNASeq) and genome-wide DNA microarrays are increasingly applied in radiation research, and have been widely employed to study the effects of radiation on humans and other mammalian species (i.e. mice, rats), however they have not yet been fully utilised in aquatic organisms (Ogawa et al. 2007; Wang et al. 2009a; Jaafar et al. 2013; Li et al. 2018). The use of high-throughput, transcriptome-wide techniques, preferably RNASeq (greater precision, sensitivity and accuracy than microarrays) should be favoured in radiation studies where applicable, data generated in such studies would allow identification of IR-specific genes, which can be utilised, or validated on a smaller-scale using qPCR (Wang et al. 2009b).

7.4.1.1 Proteomics

As noted previously in chapter 6, there has been a movement from measuring gene expression levels to proteins, as they more accurately represent the

functional molecules within a cell. The study of proteomics refers to the functional responses of gene expression; the proteins and peptides, along with protein-protein interactions (Connon et al. 2012), it allows a systems-based perspective of how proteins vary, and therefore how aquatic organisms may respond and adapt to various abiotic and biotic conditions that characterize the aquatic environment (Tomanek 2014). The potential advantage of proteomics, using 2 dimensional gel electrophoresis (2D-GE), within radiation research is not yet fully elucidated, as outlined by Leszczynski (2014) just a few studies have examined the proteome in human cells exposed to IR, with dose rates, exposure conditions and proteomics methods varying significantly from study to study.

2D-GE has been utilised to measure chemical-induced stress in aquatic organisms including marine bacteria, polychaetes, bivalves and fish (Sanchez et al. 2011; Slattery et al. 2012). While a lack of available annotated genomes and proteomes for most aquatic bivalves hinders the use of proteomic techniques (Slattery et al. 2012), marine mussels (*M. edulis*, *M. galloprovincialis*, *M. trossulus*) have gained some coverage, where changes in protein expression profiles were identified in response to a range of contaminants including flame-retardants, pharmaceuticals, metals, nanoparticles and insecticides (Dondero et al. 2010; Campos et al. 2012; Ji et al. 2013; Gomes et al. 2014a). Proteomics within ecotoxicological research has been identified as a powerful tool; it generates large amounts of meaningful data, allowing for the identification of mechanisms involved in an organism's toxicity response to environmental contaminants. Given its use, it would of interest to investigate proteomic level responses in MG and DP under the same ^{32}P exposure regimes noted in this work, enabling an understanding of IR-induced response at a more functional level than gene expression. While challenging, to fully utilise proteomic

techniques it is essential that annotated genomes for a broad diversity of aquatic organisms becomes available, particularly for ecologically relevant, or reference (RAPs) species.

7.4.1.2 Metabolomics

Metabolomics is one of the newest 'omics' technologies, it can generally be defined as the study of endogenous and exogenous low molecular mass metabolites present within a biological system (organism, cell or tissue) under a given set of conditions (Lankadurai et al. 2013). Applications to date include toxicology, agricultural research (i.e. crop breeding and plant biotechnology) and medical research including nutrition, disease diagnosis and prevention (Hall and Hardy 2012; Gomez-Casati et al. 2013). Early investigations have primarily focused on humans, plants and microbial metabolomes, through a wide spectrum of technologic methods including liquid chromatography-mass spectrometry (LC-MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR).

In more recent years the field of environmental metabolomics has emerged, where techniques are utilised to investigate complex interactions between organism and environment. Metabolomics offers several advantages over other omics technologies. Firstly, in terms of biological organisation the metabolome represents the final "omic", unlike transcripts and proteomes, metabolites represent functional, contextual entities representative of the surrounding environment (Ryan and Robards 2006). In terms of analytical approach, metabolomics provides a sensitive, high sample throughput with relative low costs, and ease of sample preparation (Miller 2007). This allows for large-scale studies, or vast sample numbers, potentially useful for determination of IR-

induced response in bivalves under field conditions (i.e. Chernobyl). Non-targeted screening of several thousand compounds, made possible via the development of high resolution mass spectrometry (HRMS) allows larger portions of the metabolome to be studied; this has potential to elucidate biomarkers for future radiological risk assessment (Lankadurai et al. 2013; Gómez-Canela et al. 2016). Lastly, metabolites are highly conserved across biological species allowing for the transferability of analytical approaches, or comparisons between aquatic biota within the same environment.

Environmental metabolomics to date has been successfully utilised in ecological relevant organisms such as freshwater crustaceans (*Gammarus pulex*, *D. magna*) and marine bivalves (*M. galloprovincialis*, *edulis*) (Taylor et al. 2009, 2010; Vandenbrouck et al. 2010; Cubero-Leon et al. 2012; Fasulo et al. 2012; Cappello et al. 2013, 2015; Nagato et al. 2013; Ji et al. 2014; Gómez-Canela et al. 2016), along with various freshwater fish (*D. rerio*, *Carassius auratus*, *Oncorhynchus mykiss*, *C. carpio*, *Odontesthes bonariensis*) (Samuelsson et al. 2006; Kullgren et al. 2010; Carriquiriborde et al. 2012; Kokushi et al. 2012; Teng et al. 2013; Xu et al. 2015).

Despite the potential of this rapidly emerging field, to our knowledge it has yet to be utilised in radiobiological research focusing on aquatic organisms. In plants (*Moringa oleifera*, *Phaseolus vulgaris*, *Arabidopsis thaliana*) however, exposure to gamma radiation (^{60}Co) was found to cause variations in metabolite distribution patterns between irradiated and non-irradiated plants (Ramabulana et al. 2015, 2016). Additionally, a study by Laiakis et al (2016) provides the first metabolomics study in urine from radiation exposed (^{137}Cs) genetic mutant animal models (*Mus musculus*). This provides evidence that this technology can be used to elucidate

effects of contaminants on metabolism by assessing bio-fluids, and in addition identify biomarkers of radiation exposure (Laiakis et al. 2016).

Despite the obvious benefits of a metabolomics approach, such technologies have their drawbacks. To determine highly diverse metabolite profiles there is a requirement for sophisticated, relatively expensive instrumentation (Gomez-Casati et al. 2013). Furthermore, a greater understanding and application of bioinformatics is required to fully interpret the large, complex data sets that metabolomics generates (Ryan and Robards 2006). Despite such challenges, the vast potential and versatility of metabolomics technologies as a routine tool for determining biological response in aquatic organisms to numerous types of environmental stressors, including radionuclides is clear. Environmental metabolomics should be viewed as complementary to other omics technologies and more classical techniques in characterizing organism response to environmental contaminants.

7.5 Conclusions

This thesis has contributed towards the elucidation of IR-induced biological responses, alone and in combination with environmental relevant Cu, in marine and freshwater mussels. Through this work, we have contributed to limited data on the chronic, low dose effects of radionuclides, using a multi-biomarker, multi-species approach, a similar approach could be adopted for other ecologically relevant species to determining biological responses. A more thorough understanding of the effects anthropogenic contaminants, such as radionuclides can have on the environment will allow the provision of more effective, inclusive radiobiological protection measures.

APPENDICES

University of Plymouth courses

- **2015:** Keeping laboratory records
- **2015:** Introduction to SPSS
- **2015:** Introduction to LaTeX

External courses

- **February 2015:** RATE Research Group Winter School, Manchester, UK.
- **September 2015:** Chernobyl Radioecology Summer School, Ukraine.
- **July 2017:** Training Course on Marine Radioactivity, as part of the Goldschmidt Conference, Paris, France.

Presentations and posters

- **November 2014:** TREE (Transfer – Exposure – Effects) research group introductory presentation, Manchester – ‘The effect of IR on marine invertebrates’
- **January 2015:** LORISE (Long-lived radionuclides in the surface environment) research meeting presentation, Manchester – ‘Assessing the impact of IR on marine invertebrates’
- **January 2015:** Plymouth University research group presentation, Plymouth – ‘Comet assay: An assay used to detect DNA strand breaks in individual cells’
- **January 2015:** Plymouth University research group presentation, Plymouth – ‘Molecular biology’

- **February 2015:** GEOWASTE research meeting presentation, Loughborough – ‘Assessing the impact of ionising radiation on aquatic organisms’
- **February 2015:** Plymouth University research group presentation, Plymouth – ‘Protein structure’
- **February 2015:** Plymouth University research group presentation, Plymouth – ‘Post-translational modification and enzymes’
- **March 2015:** Plymouth University research group presentation, Plymouth – ‘DNA repair’
- **March 2015:** Plymouth University research group presentation, Plymouth – ‘The cell cycle and DNA replication’
- **April 2015:** COGER (Co-ordinating Group for Environmental Radioactivity) conference presentation, Nottingham – ‘Assessing the impact of IR on bivalves’
- **May 2015:** TREE research group presentation, Bristol, UK – ‘Assessing the impact of IR on bivalves’
- **May 2015:** Plymouth University research group presentation, Plymouth – ‘IR and oxidative damage to DNA’
- **February 2016:** Plymouth University research group presentation, Plymouth – ‘Radioactivity in the environment’
- **April 2016:** COGER conference presentation, Glasgow – ‘Assessing the impact of IR on bivalves’
- **July 2016:** Plymouth University research group presentation, Plymouth – ‘Phosphorus-32, experimental design’
- **November 2016:** TREE Annual workshop presentation, – ‘Bioaccumulation of ^{32}P in bivalve molluscs’

- **April 2017:** COGER conference presentation, Portsmouth, UK – ‘Tissue specific bioaccumulation and release of ^{32}P in bivalve molluscs’
- **November 2017:** TREE Annual meeting presentation, Nottingham, UK – ‘ ^{32}P induced biological damage in bivalve molluscs’
- **January 2018:** RATE Final meeting poster, London – ‘Effects of radiation on aquatic invertebrates’
- **May 2018:** SETAC (Society of Environmental Toxicology and Chemistry) conference poster and presentation, Rome, Italy – ‘Tissue specific ^{32}P accumulation and consequent biological effects on bivalve molluscs’

Press releases

Dec 2015. University of Plymouth news article – ‘Analysing the fallout of radioactivity in the shadow of Chernobyl’. Mr Alan Williams.

**Radiation protection document (RPD) I: Bioaccumulation experiment
CORIF Local Rules CLR16_01**

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Local rules for the assessment of bioaccumulation of radioactivity in mussels
(*Mytilus galloprovincialis* and *Dreissena polymorpha*) chronically exposed to ^{32}P

Laboratories: Davy 110, 110A, 108, 420 & 422

1 General

Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of this experiment is to determine and compare tissue specific bioaccumulation of phosphorus-32 (^{32}P) in the marine bivalve species, *Mytilus galloprovincialis*, after chronic exposure, and to evaluate the ERICA tool's ability to accurately predict tissue concentrations of ^{32}P and determine the doses received. There will be an *in vivo* exposure of mussels to ^{32}P for 10 days (0.1, 1, 10 mGy d⁻¹), followed by the dissection and analysis of individual tissue samples using scintillation counting techniques.

The supervisor will give training in the dispensing of the radioisotopes with the aid of the Radioactive Materials Supervisor (RMS). The record keeping and monitoring of the work area will form part of the training and will be assisted, as appropriate, by the RMS. The researcher has been trained in appropriate lab technique when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and the use of a dedicated laboratory coat, gloves and safety glasses. The researcher has also been instructed as to the proper method for disposal of waste and the importance of

record keeping in line with Environment Agency requirements. Personal dosimetry, supplied via the RPA, will be worn.

2 Area description

Dilutions of ^{32}P from the stock solution will be performed in a fume cupboard, designated for use with radioactive materials, in Room 314c of the Davy Building by the RMS, Nick Crocker. Exposure of mussels will be carried out in a controlled temperature room (CR2) adjacent to Davy 420. This area is designated an 'other area' in the PU Radiation Safety Handbook, therefore maximum total activity at one time must be $<1/10$ th annual limit on intake (ALI). ALI for ^{32}P is 8.3 MBq for ingestion and 6.3 MBq for inhalation, in accordance with the Plymouth University radiation safety document 2011. This equates to the ICRP limit of effective dose at 100 mSv in a 5 year period (20 mSv per year). The maximum total activity permitted in CR2 is 0.63 MBq (630000 Bq), in accordance to the most stringent ALI (inhalation).

The room will be cordoned off, locked when not in use, and clearly labelled with trefoil as containing radiological material for the duration of the experiment. It is impractical to carry out this work in Davy 110, due to the need for a controlled temperature room (both species) with seawater on tap (*Mytilus* spp.) and a set photoperiod. Dissection will also take place in CR2, to minimise transport of contaminated animals. Further processing of tissue samples (e.g. digestion of tissues into cell suspensions) will take place in Davy 110 or 314c. Preparation for scintillation counting will be conducted in Davy 314c and CR2, with analysis at the University of Portsmouth. For disposal, ^{32}P treated water will be transported in sealed plastic containers (polypropylene, minimum thickness 1cm), within an additional larger plastic box, to the sink in Davy 110 designated for the disposal

of radioactive liquids. Accurate records will be kept of all disposals in the laboratory record sheets and any discrepancies reported immediately to the RMS. All potentially radioactive areas and areas where radioactive materials are present will be noted on a hazard map within the door of CR2.

Experiments are designed to ensure that researcher doses are as low as reasonably practical. The total activity and dose in the laboratory at any one time during the experiment will be 515536 Bq (0.515536 MBq - including WSA during dosing of beakers), this is under the maximum total activity permitted in CR2 of 0.63 MBq, and equivalent to a total worker dose to EV over the duration of the experiment (including WSA, exposure, sampling and transportation) of 0.835 mSv.

3 Dose investigation levels

ATP $\gamma^{32}\text{P}$ will be purchased from Perkin-Elmer in batches of 15 MBq (the smallest available – total activity varies per batch). In this form its specific activity is 600 MBq/ml and therefore the volume of each received batch is calculated as 25 μl . In accordance with ERICA tool predicted dose rates, a total activity of 236097 Bq for *M. galloprovincialis* will be required for the experiment. Considering the half-life (14.29 days) of ^{32}P , we will need to purchase 1 batch for the duration this experiment. The university's storage limit for 'any other radionuclide except alpha emitter (in total)' is 1 GBq, which includes ^{32}P .

All ^{32}P stock solutions will be stored and controlled by the RMS, Nick Crocker, (Room 314a, Davy Building). The RMS will be consulted to ensure that holdings of ^{32}P do not exceed the storage limit, at the time of ordering, when added to the other radionuclides already present. Stock record paperwork will be completed when aliquots of the radioactive solution are removed from storage for use and

will be reconciled with disposal records at the end of every batch run. Key elements of record keeping are: (i) Isotope store stock record, completed and kept in Davy 314c, which will detail the remaining stock solution for each batch purchased (decay corrected to certified date); (ii) CT room current holdings record, completed and kept in CT2, will contain details of the activity currently present in the cold room; (iii) the disposal record in 110 will be maintained and reconciled with stock and CT room records at the end of each batch run and reported to the RMS prior to next aliquot being drawn. All record sheets will be returned to Nick Crocker on completion of the experiment.

3.1 Dose from storage stock (NC)

For the *M. galloprovincialis* bioaccumulation experiment: The main stock (11.79 MBq/25 µl [delivery date - 12.12.16] – 3.5 MBq on day of use [05.01.17]) will be divided into two stocks the day before the experiment, a storage stock (2.1 MBq/15 µl) and working stock A (1.4 MBq/10 µl), both to be dispensed by Nick Crocker (NC) in Davy 314c. Worker dose will be based on (a) proximity to storage stock and (b) pipetting of storage stock. To note, pipetting of stock will be performed behind a perplex screen in a fume hood, finger dosimeters will be worn. Total maximum dose from the main stock (including dose from dispensing WSA into 2 ml aliquots [see section 3.1], not including accidental spillage) is 0.512 mSv.

3.1.1 Proximity to storage stock

- Total activity of solution in use: 3492997.619 Bq (3.5 MBq)
- Dose rate (mSv hr⁻¹) and pathway: Infinite plane source (1 m) – 0.168 mSv h⁻¹
 - 1 MBq = 0.048 mSv for 1 h

- MBq = 0.168 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 30 s exposure, once during experiment
- Total dose for experimental step (mSv): 0.0014 mSv – $[(0.168/60)/2]$

3.1.2 Pipetting of storage stock and accidental spillage on skin

- Total activity of solution in use: 3492997.619 Bq (3.5 MBq)
- Dose rate (mSv h⁻¹) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 3.5 MBq = 83.65 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 10 s exposure, once during experiment
- Total dose for experimental step (mSv): 0.232 mSv $[(83.65/60)/6]$
- Consequence of accidental spillage on skin (total dose): 6.4652 mSv (10 s) – to note it is highly unlikely the entire stock volume would be on skin
 - 0.05 ml 1 kBq droplet = 1.33 mSv h⁻¹
 - 0.05 ml 3.5 MBq droplet = 4655 mSv h⁻¹ $[(1.33*1000)*3.5]$
 - 0.025 ml (stock volume, 25 µl) 3.5 MBq droplet = 2327.5 mSv h⁻¹
 - 0.025 ml 3.5 MBq droplet = 6.465 mSv (10 s)

3.2 Worker dose: Experimental researcher (EV dose – unless stated)

3.2.1 Dose from working stock

Working stock solution (working stock A, WSA, total activity 1397199.04 Bq/10 mL) taken into Davy 420 CR2 for experimental use will not exceed a total activity of 279439.8 Bq/2 mL (0.27 MBq). Worker dose calculations will be based on (a) dispensing of WSA by NC into 2 mL tubes, (b) proximity to WSA and (c) pipetting

of WSA into beakers. The total dose to NC from WSA will be 0.278 mSv. Total dose from working stock (including whole body and extremity dose (pipetting) for EV will be 0.558 mSv.

3.2.2 Dispensing of WSA into 2 mL aliquots (NC dose)

- Total activity of solution in use: 1397199.04 Bq
- Dose rate (mSv h⁻¹) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 1397199.04 Bq = 33.393057056 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 30 s exposure, once
- Total dose for experimental step: 0.278275475 mSv – [(33.393057056 /60)/2]

3.2.3 Pipetting of WSA into beakers

- Total activity of solution in use: 279439.8 Bq
- Dose rate (mSv h⁻¹) and pathway: Contact with 5 mL plastic syringe -
 - 1 MBq = 23.9 mSv for 1 h
 - 279439.8 Bq = 6.67861122 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 1 min, five times within the experiment – total 5 min
- Total dose for experimental step (mSv): 0.556550935 mSv – [6.67861122/12]

3.2.4 Proximity to WSA (WSA stored in two plastic boxes to reduce exposure)

- Total activity of solution in use: 279439.8 Bq
- Dose rate (mSv h⁻¹) and pathway: 1 m infinite plane source

- $1 \text{ MBq} = 0.048 \text{ mSv for 1 h}$
- $279439.8 \text{ Bq} = 0.01341311 \text{ mSv for 1 h}$
- Total duration of exposure and number of times within the experiment: 1 min, five times within the experiment – total 5 min
- Total dose for experimental step (mSv): $0.001117759 \text{ mSv} - [0.01341311/12]$

3.2.5 Dose from glass beakers – including water sampling

Worker dose calculations will be based on (a) proximity to beakers whilst shielding lid is open (b) pipetting whilst taking water samples, these (1 mL in duplicate) will be taken on days 1, 5 and 9. Pipetting and contact with glass beakers will be kept to a minimum, efforts will be made to reduce this via acrylic shielding. In addition, all dispensing will be carried out in bench-kote lined trays. Dose to finger extremities and chest will be monitored using a dose meter, before, during and after the exposure. Total dose from glass beakers (including whole body and extremity dose (pipetting) for EV will be 0.0754 mSv .

3.2.5.1 Proximity to beakers during water changes

- Total activity of solution in use: 236097 Bq
- Dose rate (mSv h^{-1}) and pathway: 1 m infinite plane source
 - $1 \text{ MBq} = 0.048 \text{ mSv for 1 h}$
 - $236097 \text{ Bq} = 0.011332656 \text{ mSv for 1 h}$
- Total duration of exposure and number of times within the experiment: 30 min, five times within the experiment – total 2.5 h
- Total dose for experimental step: $0.02833164 \text{ mSv} [0.011332656 * 2.5]$

3.2.5.2 Pipetting whilst taking water samples

Samples will be stored in plastic container in isotope store (to note- each beaker is covered individually therefore worker will not be exposed to total activity of beakers all at once). Exposure time was decreased to account for this.

- Total activity of solution in use: 236097 Bq
- Dose rate (mSv h^{-1}) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 236097 Bq = 5.6427183 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 10 s, three times within the experiment – total 30 s
- Total dose for experimental step: 0.047022653 mSv – $[(5.6427183/60)/2]$

3.2.6 Dose from handling radioactive mussels

Worker dose calculations are based on proximity to radioactive mussels. *M. galloprovincialis* were found to uptake approximately 54% of ^{32}P , therefore total activity in samples is estimated at 127492.38 Bq – to note, worker will not be exposed to all mussels at same time, therefore worker dose in reality will be far lower. Total dose from handling radioactive mussels (including whole body and extremity dose (pipetting) for EV will be 0.150 mSv.

- Total activity of solution in use: 127492.38 Bq
- Dose rate (mSv h^{-1}) and pathway: Point source (30 cm)
 - 1 MBq = 0.118 mSv for 1 h
 - 127492.38 Bq = 0.015044101 mSv for 1 h
- Total duration of exposure and number of times within the experiment: Total sampling procedure time – 10 h once at end of exposure
- Total dose for experimental step: 0.15044101 mSv – $[0.015044101 * 10]$

3.2.7 Dose from transportation of samples

Worker dose calculations will be based on proximity to water and tissue samples. Water samples (90 total) will have a maximum activity of 236 Bq. This is calculated from the following: 15 (1 mL in duplicate – 2 per beaker) water samples taken on days 1, 5 and 9 – totalling 472 Bq (not accounting for decay), this is divided by 2 with the assumption that 54% of ^{32}P (in accordance to collected data) will be absorbed into mussel tissue. Tissue (shell, soft tissue and IMW) samples (585 total) will have a maximum activity 118048.5 Bq (approx.), assuming that approximately 54% of ^{32}P has been absorbed into the mussels, and 10% of each individual will be utilised for LSC. Total dose from the transportation of samples (including whole body and extremity dose (pipetting) for EV will be 0.0511 mSv.

3.2.7.1 Proximity to water samples during transportation

- Total activity of solution in use: 236 Bq
- Dose rate (mSv h^{-1}) and pathway: 1 m infinite plane source
 - 1 MBq = 0.048 mSv for 1 h
 - 236 Bq = 0.000011328 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 9 h driving to/from the University of Portsmouth
- Total dose for experimental step (mSv): 0.000101952 mSv [0.000011328×9]

3.2.7.2 Proximity to tissue samples during transportation

- Total activity of solution in use: 118048.5 Bq
- Dose rate (mSv h^{-1}) and pathway: 1 m infinite plane source
 - 1 MBq = 0.048 mSv for 1 h
 - 118048.5 Bq = 0.005666328 mSv for 1 h

- Total duration of exposure and number of times within the experiment: 9 h driving to/from the University of Portsmouth
- Total dose for experimental step: 0.050996952 mSv [0.005666328*9]

3.2.8 Total dose rate

The total annual dose rate to EV from the *M. galloprovincialis* bioaccumulation experiment is 0.835 mSv, well under the 20 mSv per year limit. To note, this is also well under the limit for women of reproductive capacity, where an equivalent dose limit of 13 mSv in any consecutive period of 3 months applies. EV will keep a record of accumulated dose for this and other related experiments to inform future Local Rules as required.

4 Working instructions

4.1 Methods

All reagents will be kept in Room 422 and radioactive materials will be clearly marked with trefoil warning signs on vessels and spill trays. Beakers, acrylic 12 mm shielding, air-stones, air pumps, electronic siphon hoses, water containers, plastic storage boxes, stirrers and volumetric flasks will be kept in room 420 CR2. The tray, pipettes, pipette tips and plastic gloves used will be kept in Room 110 and CR2, these will be double bagged and labelled in CR2 and brought to the bins in Davy 110 for disposal. LSC will be conducted using the scintillation counter at the University of Portsmouth (see section for 6 details). Weighing will be conducted using the balances in Rooms 110 and 422.

4.2 Exposure scenario

Three *M. galloprovincialis* mussels per labelled beaker (1 L) will be exposed to the following activity concentrations of ^{32}P in triplicate: 709, 7090 or 70900 Bq L⁻¹, along with control and ATP (phosphorus only) treatments (15 beakers total). Each beaker will be aerated via tubing fit through individual, circular acrylic beaker covers. Dilutions of concentrated stock solution taken from Davy 314C will be made with deionised water in CR2, obtained from taps in Davy 420. The exposure experiments will take place over a period of 10 d, with 5 x half water changes (500ml, on days 1, 3, 5, 7, 9). Siphoning will be performed using an electric siphon which does not require holding during use, this will minimise operator exposure. At each water change and on the final day the contaminated water will be drained by siphoning the water directly into a large (50 L), sealable plastic container (carboy) and taken to Room 110 for disposal. The electronic siphon will be cleaned between water changes by transferring clean water into the carboy to rinse out any remaining ^{32}P . This water will be treated as contaminated and disposed of with the beaker water in the designated sink in Davy 110. ^{32}P concentration in exposure vessels (beakers) will then be topped up using the appropriate stock. On day 10, the exposure will finish so there will be no renewal of ^{32}P in that specific experiment. Water samples (1 mL) will be taken on day 1, 5 and 10 for liquid scintillation counting to determine water activity concentrations during the experiment. This exposure scenario results in a maximum activity of 236097 Bq (0.236097 MBq) in Davy 420 CR2 at any one time, which is lower than 1/10th total ALI (0.63 MBq - 630000 Bq) and below the maximum recommended bench top activity (2 MBq). Following the exposure, glassware (i.e. beakers) will be rinsed 3 times with DI water, this will be siphoned off into the sealable plastic container (carboy) and taken to Room 110 for

disposal. Beakers will then be taken down to room 110 for a further rinse; they will then be acid washed in room 422. All working areas will be monitored for contamination before and after all radioactive exposures, suitable records will be kept.

5 Sampling

At the end of the exposure period, mussels (45 individual's total) will be dissected into their soft tissue (adductor muscle, digestive gland, mantle, gills and 'other') and shell, internal water will be drained directly into scintillation vial (in CR2) for determination of ^{32}P activity, as detailed below. After LSC analysis, any waste mussel samples will be placed in a sealed plastic carboy in Davy 314C, this will then be stored for 3/4 months before disposal as non-hazardous lab waste in yellow bags, in accordance with the radioactive substance act 1993. All waste will be checked with suitable detector prior to disposal. This will remain well below the university storage limits of 1 GBq.

5.1 Determination of radioactivity within mussel tissues

Mussels will be dissected into soft tissue (adductor muscle, digestive gland, mantle, gills and 'other'), shell and internal mussel water. Soft tissue will be placed into pre-weighed falcon tubes, re-weighed and homogenised in 10 mL DI water. 1 mL samples will be aliquoted (in duplicate) into a scintillation vial and mixed with 4 mL scintillation cocktail (LabLogic U). Mussel shells will be crushed using a hammer and pestle and mortar, and placed into pre-weighed falcon tubes. After re-weighing, shells will be solubilised in concentrated nitric acid (< 5 hr). Following solubilisation, 1 mL solution will be added to 4 mL scintillation cocktail. 4 mL scintillation cocktail is added to water samples and internal mussel water.

6 Transportation of samples to the University of Portsmouth, St Michael's building

Samples, in individual scintillation vials will be packed in scintillation boxes and placed in sealed plastic bags, labelled with trefoil warning labels, surrounded by blue roll. A layer of vermiculite will line the bottom of the box as a precaution. This will then be placed into sealed, plastic boxes with warning labels on the inside. Water samples (90 total) will have a maximum radiation activity of 236 Bq. This is calculated from the following: 15 (1 mL in duplicate – 2 per beaker) water samples taken on days 1, 3 and 5 – totalling 472 Bq (not accounting for decay), this is divided by 2 with the assumption that 54% of ^{32}P (in accordance to collected data) will be absorbed into mussel tissue. Tissue (shell, soft tissue and internal mussel water) samples (585 total) will have a radiation level of approximately 118048.5 Bq. This is assuming that approximately 54% of ^{32}P has been absorbed into the mussels, and 10% of each individual will be utilised for LSC. Maximum total will therefore be 11804.85 Bq.

In accordance with the Regulations for the safe transport of radioactive material, SSR6 (2012 edition), which state that the activity limit for an exempt consignment (^{32}P specific) is 100000 Bq, samples will be transported to University of Portsmouth by the researcher via car. Following LSC at the University of Portsmouth (St Michael's building, small instrument room), (Hidex 300 SL, allows analysis of low radioactivity levels in samples) the data will be decay adjusted, and converted to Bq g^{-1} using the weight of the samples and to a dose rate in $\mu\text{Gy h}^{-1}$ using wet weights and the ERICA tool. Samples will be brought back to

Plymouth and disposed of as shown below. In regards to transportation, the driver will;

1. be aware of contents and will know what actions to be taken in the unlikely event of a spill,
2. ensure contact details and copy of the Local Rules are available with the package for the emergency services should they not be able to talk with the driver,
3. ensure relevant source records are updated,
4. monitor packages for both surface dose rate and contamination before dispatch and recording the values,
5. produce a document to accompany the package that details the contents,
6. ensure that there is sufficient data that proves the activity levels are within the exempt limits stated,
7. ensure that the receiving facility has the necessary Permits and facilities to keep and work with the material,
8. ensure the RPS is aware of the proposals and comments as necessary.

7 Disposals

The entire experimental procedure for the researcher to be undertake under these LRs is noted above. The exposure scenario described above will result in a maximum total disposal activity of 236097 Bq ^{32}P (in 684 individual scintillation vials [max 118048.5 Bq] and via water waste [max 118048.5 Bq]). This is within the university's monthly aqueous disposal limit for ^{32}P of 50 MBq. Maximum theoretical disposal for the entire experimental procedure covered by these Local Rules (assuming one batch of standard solution as defined in section 3 is purchased per experiment and entire stock disposed of each time) would be 15

MBq, which is still well under this limit. Small amounts of solid radioactive waste (waste tissue), sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be stored in a plastic bag in the 314C freezer for around two months (4.5 half-lives) before disposal as solid waste in Davy 110. The levels of activity associated with the solid waste will be below the bi-monthly disposal limit of 40 KBq (The sum total of kBq of all other radionuclides in any one item of waste does not exceed 40). All disposals will be recorded and decay corrected to the certificate date to ensure records are accurate and comparable.

Disposals will be made of analysed solutions at the end of each experimental batch run. New experiments will not be started until all disposals from prior experiment have been undertaken and appropriate records made and approved by the RMS and CORIF laboratory manager (Dr Alex Taylor). Under no circumstances, will samples that have been analysed be stored in cupboards or bench space in the Davy 108, 110, 110A laboratory suite.

8 Restricting exposure

Potential exposure from this experiment is low. All siphoning will be performed using an electronic siphon so there will be nil by mouth and no prolonged exposure to limbs or digits. Therefore, the amount of ^{32}P ingested will be nil and considerably below the 20 mSv ALI ingestion of 8.3 MBq (see section 3.1). As noted from previous experiments, there is limited evaporative loss. Beakers are covered with circular acrylic sheets/covers whilst within the shielding which eliminates evaporative loss or contamination of the controlled temperature room. This effectively reduces the evaporative loss (and associated dose) to zero. The acrylic covers will only be removed during siphoning/refilling during which time the operator will be at a distance from the beakers. After use, circular covers will

be rinsed (water directly siphoned into wastewater carboy) and stored in the acrylic shielding. The proposed safety practices should be sufficient in maintaining a safe working environment for the researchers. It is necessary to exercise extreme care when handling the radioactive material, especially when in the concentrated state prior to dilution. The safety record will be signed by the Radiation Protection Supervisor (RPS) following each exposure. The researchers will wear a designated red lab coat, safety glasses and gloves at all times when working in Rooms 110, 110a and 420 CR2 (and a mask in the constant temperature room). The RMS or researcher (student) will take three swabs before and after each exposure to assess any increases in activity above typical levels of the surfaces in the constant temperature room, activity at background level is considered acceptable. The record of these measurements and indication of that areas were 'All Clear' on assessment (and/or remedial action taken) will be kept in a visible location in the laboratory.

9 Contingency arrangements

During the exposure period and at all water changes the beakers will be kept within specially made 1 cm thick acrylic shielding, designed to contain all contents if a leak should occur. In case of any spillages a vermiculite spill kit will be kept in the lab, this will be used when any spill of liquid thought to contain radioactive material is encountered, the area should then be cleaned with a detergent and copious amounts of water. Any spillage onto skin will be treated by immersing contaminated area into saturated potassium permanganate solution (this will be kept in CR2), followed by a rinse to decolourise it with 5% sodium sulphite solution. This treatment will remove the dead cell layer containing the ^{32}P , skin should not be rubbed vigorously with a hard brush, the skin may be damaged and

contamination could enter the bloodstream. Area, equipment (Including lab coat, glasses etc.) will be tested through swabbing for any residual contamination. The emergency protocols detailed in the handbook will be applied, including minor accidents and fire risk, essential safety procedures will be printed and readily available in the CR2.

10 Radiological safety contacts

The Radiation Protection Advisor (RPA), available 24/7 for emergencies, is:

Cliff Ellis (BSc, MSc, MSRP)

HP2 Radiation Protection Services (www.hp2radiationprotectionservices.co.uk/)

Office: 01305 858506

Mobile: 07786 405769

Emergency Bleep: 07623 971247

Backup RPA: Robert Truman mobile 07786 405767

The Radiation Protection Supervisor (RPS) is:

Professor William Blake,

School of Geography, Earth & Environmental Sciences

University of Plymouth

Drake Circus, Plymouth PL4 8AA

tel. +44 (0)1752 585969

Email: William.Blake@plymouth.ac.uk

The Deputy Radiation Protection Supervisor is:

Dr Alex Taylor

SoGEES,

Plymouth University

Tel: 01752 585940

The Radioactive Materials Supervisor is:

Mr. N. Crocker

School of Biomedical & Biological Sciences

Faculty of Science and Technology

University of Plymouth

Drake Circus

Plymouth

Devon

PL4 8AA

Tel: 01752 232928

Fax: 01752 232927

Email: N.Crocker-1@plymouth.ac.uk

All emergency contact details are held by Security.

11 Supporting information

Appropriate Risk Assessment/COSHH forms approved by the relevant authority must be attached to validate Local Rules.

Radiation protection document II: Biological end-point experiments
CORIF Local Rules CLR16_02

Researcher: Miss E. L. Vernon
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Tel: 07764486133
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Date: April 2017

Supervisors: Professor A. Jha
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Local rules: to determine the genotoxic and molecular responses of adult
freshwater (*D.polymorpha*) bivalves following exposure to ^{32}P

Laboratories: Davy 110, 110A, 108, 420 & 422

1 General

Local Rules are provided in accordance with Regulation 17(1) of the Ionising Radiations Regulations 1999 (IRR99). The aim of this experiment is to determine and compare tissue specific genetic and molecular responses in *Dreissena polymorpha* and *Mytilus galloprovincialis* (this local rules refers to the *D. polymorpha* experiment due to higher activity levels), after chronic exposure to phosphorus-32 (^{32}P). There will be an *in vivo* exposure of mussels to ^{32}P for 10 days (0.1, 1, 10 mGy d⁻¹).

The supervisor will give training in the dispensing of the radioisotopes with the aid of the Radioactive Materials Supervisor (RMS). Record keeping and monitoring of work area will form part of the training and will be assisted, as appropriate, by the RMS. The researcher has been trained in appropriate laboratory techniques when handling radioactive materials, e.g. making sure that spills are dealt with effectively, clear labelling of samples so that co-workers are fully aware of the presence of any radioactive substances and use of a dedicated laboratory coat, gloves and safety glasses. The researcher has also been instructed as to the proper method for disposal of waste and the importance of

record keeping in line with EA requirements. Personal dosimetry, supplied via the RPA, will be worn.

2 Area description

Dilutions of ^{32}P from the stock solution will be performed in a fume cupboard, designated for use with radioactive materials, in Room 314c of the Davy Building by the RMS, Nick Crocker. Exposure of mussels will be carried out in a controlled temperature room (CR2) adjacent to Davy 420. This area is designated an 'other area' in the PU Radiation Safety Handbook, therefore maximum total activity at one time must be $<1/10^{\text{th}}$ annual limit on intake (ALI). ALI for ^{32}P is 8.3 MBq for ingestion and 6.3 MBq for inhalation, in accordance with the University of Plymouth radiation safety document 2011. This equates to the ICRP limit of effective dose at 100 mSv in a 5 year period (20 mSv per year). The maximum total activity permitted in CR2 is 0.63 MBq (630000 Bq), in accordance to the most stringent ALI (inhalation).

The room will be cordoned off, locked when not in use, and clearly labelled with trefoil as containing radiological material for the duration of the experiment. It is impractical to carry out this work in Davy 110, due to the need for a controlled temperature room (both species) with seawater on tap (*M. galloprovincialis*) and a set photoperiod. Dissection will also take place in CR2, to minimise transport of contaminated animals. Further processing of tissue samples (e.g. digestion of tissues into cell suspensions) will take place in Davy 110 or 314c. Preparation for scintillation counting will be conducted in Davy 314c and CR2, with analysis at the University of Exeter.

For disposal, ^{32}P treated water will be transported in sealed plastic containers (polypropylene, minimum thickness 1cm), within an additional larger plastic box, to the sink in Davy 110 designated for the disposal of radioactive liquids. Accurate records will be kept of all disposals in the laboratory record sheets and any discrepancies reported immediately to the RMS. All potentially radioactive areas and areas where radioactive materials are present will be noted on a hazard map within the door of CR2.

Experiments are designed to ensure that researcher doses are as low as reasonably practical. Total activity and dose in the laboratory at any one time during the experiment will be 374625 Bq (0.374625 MBq – not including stock) or 0.559625 MBq (including WSA [0.185 MBq] during dosing of beakers). This is under the maximum total activity permitted in CR2 of 0.63 MBq, and equivalent to a worker dose to EV over the duration of the experiment (including WSA, exposure, sampling and transportation) of 0.56 mSv.

3 Dose investigation levels

ATP $\gamma^{32}\text{P}$ will be purchased from Perkin-Elmer in batches of 15 MBq (the smallest available – total activity varies per batch). In this form its specific activity is 600 MBq/mL and therefore the volume of each received batch is calculated as 25 μL . In accordance with ERICA tool predicted dose rates, a total activity of 374625 Bq for *D. polymorpha* will be required for the experiment. Considering ^{32}P 's half-life we will need to purchase 1 batch for the duration this experiment. The university's storage limit for 'any other radionuclide except alpha emitter (in total)' is 1 GBq, which includes ^{32}P .

All ^{32}P stock solutions will be stored and controlled by the RMS in Room 314a (Davy Building). The RMS will be consulted to ensure that holdings of ^{32}P do not exceed the storage limit at the time of ordering, when added to the other radionuclides already present. Stock record paperwork will be completed when aliquots of the radioactive solution are removed from storage for use and will be reconciled with disposal records at the end of every batch run. Key elements of record keeping are: (i) Isotope store stock record, completed and kept in Davy 314c, which will detail the remaining stock solution for each batch purchased (decay corrected to certified date); (ii) CT room current holdings record, completed and kept in CT2, will contain details of the activity currently present in the cold room; (iii) the disposal record in 110 will be maintained and reconciled with stock and CT room records at the end of each batch run and reported to the RMS prior to next aliquot being drawn. All record sheets will be returned to the RMS on completion of the experiment.

3.1 Dose from storage stock (NC)

For *D. polymorpha* genetic/molecular experiments: Main stock (Approx. 9.25 MBq/25 μl (varies on delivery)) will be divided into four stocks one day prior to exposure;

1. Storage stock - 1.85 MBq/5 μl
2. Working stock A (for 10 mGy d^{-1} treatment) – 3.7 MBq/10 mL (aliquots taken into CR2 to ensure total activity is below 0.63 MBq)
3. Working stock B (for 0.1 and 1 mGy d^{-1} treatment) - 3.7 MBq/1 mL
4. Working stock B.1 (for 0.1 and 1 mGy d^{-1} treatment) – 0.37 MBq/10 mL

Worker dose will be based on (a) proximity to storage stock and (b) pipetting of storage stock. To note, pipetting of stock will be performed behind a perplex screen in a fume hood, finger dosimeters will be worn. Pipetting of all stocks will be carried out with a pipette, not a plastic syringe. Calculated dose rates from 'contact with 5 mL plastic syringe' are therefore far greater. Total maximum dose from the main stock (including dose from dispensing WSA into 0.5 mL aliquots [see section 3.1], not including accidental spillage) is 1.3547 mSv (in reality far lower due to use of pipette).

3.1.1 Proximity to storage stock

- Total activity of solution in use: 9250000 Bq (9.25 MBq)
- Dose rate and pathway: Infinite plane source (1 m) – 0.444 mSv h⁻¹
 - 1 MBq = 0.048 mSv for 1 h
 - 9.25 MBq = 0.444 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 30 s exposure, once during experiment
- Total dose for experimental step: 0.0037 mSv – [(0.444/60)/2]

3.1.2 Pipetting of storage stock and accidental spillage on skin

- Total activity of solution in use: 9250000 Bq (9.25 MBq)
- Dose rate (mSv h⁻¹) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 3.5 MBq = 221.075 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 10 s exposure, once during experiment
- Total dose for experimental step: 0.614 mSv [(221.075/60)/6]

- Consequence of accidental spillage on skin (total dose): 6.4652 mSv (10 s) – to note it is highly unlikely the entire stock volume would be on skin
 - 0.05 mL 1 kBq droplet = 1.33 mSv h⁻¹
 - 0.05 mL 9.25 MBq droplet = 12302.5 mSv h⁻¹ [(1.33*1000)*9.25
 - 0.025 mL (stock volume, 25 µL) 9.25 MBq droplet = 6151.25 mSv h⁻¹
 - 0.025 mL 9.25 MBq droplet = 17.09 mSv (10 s)

3.2 Worker dose: Experimental researcher (EV dose – unless stated)

3.2.1 Dose from working stock

Working stock solutions (Includes working stock A and B and B.1, total activity 3700000 Bq/10 mL) taken into Davy 420 CR2 for experimental use will not exceed a total activity of 185000 Bq/0.5 mL (0.185 MBq). Worker dose calculations will be based on (a) dispensing of WSA by NC into 0.5 mL tubes, (b) proximity to WSA and (c) pipetting of WSA into beakers. Total dose to NC from WSA will be 0.737 mSv. Total dose from working stock (including whole body and extremity dose (pipetting) for EV will be 0.75 mSv.

3.2.2 Dispensing of WSA into 0.5 ml aliquots (NC dose)

- Total activity of solution in use: 3700000 Bq (3.7 MBq)
- Dose rate (mSv hr⁻¹) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 3.7 MBq = 88.43 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 30 s exposure, once
- Total dose for experimental step: 0.737 mSv – [(88.43/60)/2]

3.2.3 Pipetting of WSA aliquot into beakers

- Total activity of solution in use: 185000 Bq
- Dose rate (mSv h⁻¹) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 185000 Bq = 4.4215 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 1 min, five times within the experiment – total 5 min
- Total dose for experimental step: 0.368458333 mSv – [4.4215/12]

3.2.4 Proximity to WSA

- WSA aliquots stored in two plastic boxes to reduce exposure
- Total activity of solution in use: 185000 Bq
- Dose rate (mSv h⁻¹) and pathway: 1 m infinite plane source
 - 1 MBq = 0.048 mSv for 1 h
 - 185000 Bq = 0.00888 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 1 min, five times within the experiment – total 5 min
- Total dose for experimental step: 0.00074 mSv – [0.00888/12]

3.2.5 Dose from glass beakers (Including water sampling)

Worker dose calculations are based on (a) proximity to beakers whilst shielding lid is open, and (b) pipetting whilst taking water samples (1 mL in duplicate), taken on day 1 and 9. Pipetting and contact with glass beakers will be kept to a minimum, efforts will be made to reduce this via acrylic shielding. In addition, all dispensing will be carried out in bench-kote lined trays. Dose to finger extremities and chest will be monitored using a dose meter, before, during and after the

exposure. Total dose from glass beakers (including whole body and extremity dose (pipetting) for EV will be 0.11956781 mSv.

3.2.5.1 Proximity to beakers during water changes

- Total activity of solution in use: 374625 Bq
- Dose rate (mSv h⁻¹) and pathway: 1 m infinite plane source
 - 1 MBq = 0.048 mSv for 1 h
 - 374625 Bq = 0.017982 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 30 min, five times within the experiment – total 2.5 h
- Total dose for experimental step: 0.044955 mSv [0.017982 *2.5]

3.2.5.2 Pipetting whilst taking water samples

Samples will be stored in plastic container in isotope store (to note- each beaker is covered individually therefore worker will not be exposed to total activity of beakers all at once –exposure time is decreased to try and account for this.

- Total activity of solution in use: 374625 Bq
- Dose rate (mSv h⁻¹) and pathway: Contact with 5 mL plastic syringe
 - 1 MBq = 23.9 mSv for 1 h
 - 374625 Bq = 8.9535375 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 10 s, three times within the experiment – total 30 s
- Total dose for experimental step: 0.074612813 mSv – [(8.9535375/60)/2]

3.2.6 Dose from handling radioactive mussels

Worker dose calculations are based on (a) proximity to radioactive mussels. From previous experiments *D. polymorpha* were found to uptake approximately 17% of

^{32}P , therefore the total activity in the samples will be estimated at 63686.25 Bq.

To note, worker will not be exposed to mussels at the same time, therefore dose in reality will be far lower. Total dose from handling radioactive mussels (including whole body and extremity dose (pipetting) for EV will be 0.075149775 mSv.

- Total activity of solution in use: 63686.25 Bq
- Dose rate (mSv h^{-1}) and pathway: Point source (30 cm)
 - 1 MBq = 0.118 mSv for 1 h
 - 63686.25 Bq = 0.007514978 mSv for 1 h
- Total duration of exposure and number of times within the experiment: Total sampling procedure time – 10 h, end of exposure
- Total dose for experimental step: 0.075149775 mSv – $[0.007514978 \times 10]$

3.2.7 Dose from transportation of samples

Worker dose calculations will be based on proximity to water samples. Water samples (50 total) will have a maximum radiation activity of 999 Bq. This is calculated from the following: 12 (1 mL in duplicate, two per beaker) water samples taken on days 1 and 9. Total dose from the transportation of samples for EV will be 0.000191808 mSv.

3.2.7.1 Proximity to water samples during transportation

- Total activity of solution in use: 999 Bq
- Dose rate (mSv h^{-1}) and pathway: 1 m infinite plane source
 - 1 MBq = 0.048 mSv for 1 h
 - 999 Bq = 0.000047952 mSv for 1 h
- Total duration of exposure and number of times within the experiment: 4 h
- Total dose for experimental step: 0.000191808 mSv $[0.000047952 \times 4]$

3.2.8 Total dose rate

Total annual dose rate to EV from the *D. polymorpha* genetic/molecular experiment (including WSB stock solutions, exposure, sampling and transportation) is 0.564107729 mSv, well under the 20 mSv per year limit. To note, this is also well under the limit for women of reproductive capacity, where an equivalent dose limit of 13 mSv in any consecutive period of 3 months applies.

4 Working instructions

4.1 Methods

All reagents will be kept in Room 422 and radioactive materials will be clearly marked with trefoil warning signs on vessels and spill trays. Beakers, acrylic 12 mm shielding, air-stones, air pumps, electronic siphon hoses, water containers, plastic storage boxes, stirrers and volumetric flasks will be kept in Room 420 CR2. The tray, pipettes, pipette tips and plastic gloves used will be kept in Room 110 and CR2, and these will be double bagged and labelled in CR2 and brought to the bins in Davy 110 for disposal. Scintillation counting will be conducted using the scintillation counter at the University of Exeter (see section for 6 details). Weighing will be conducted using balances in Rooms 110 and 422.

4.2 Exposure scenario

D. polymorpha mussels (total weight 17.5 g, ~7 individuals) per labelled beaker (500 mL) will be exposed to the following activity concentrations of ^{32}P in triplicate: 2250, 22500 or 225000 Bq L⁻¹, along with control, and Cu (positive control - 56 µg L⁻¹) treatments (15 beakers total). Each beaker will be aerated via tubing fit through individual, circular acrylic beaker covers. Dilutions of concentrated stock solution taken from Davy 314C will be made with DI water in

CR2, obtained from taps in Davy 420. The exposure will take place over a period of 10 d, with 4 x half water changes (250 mL, on days 3, 5, 7, 9). Siphoning will be performed using an electric siphon which does not require holding during use, this will minimise operator exposure. At each water change and on the final day the contaminated water will be drained by siphoning the water directly into a large (50 L), sealable plastic container (carboy) and taken to Room 110 for disposal. The electronic siphon will be cleaned between water changes by transferring clean water into the carboy to rinse out any remaining ^{32}P . This water will be treated as contaminated and disposed of with the beaker water in the designated sink in Davy 110. The ^{32}P concentration in exposure vessels (beakers) will then be topped up using the appropriate stock. On day 10, the exposure will finish so there will be no renewal of ^{32}P in that specific experiment. Water samples (1 mL – in duplicate) will be taken on days 1 and 9 for liquid scintillation counting to determine water activity concentrations. This exposure scenario results in a maximum activity of 0.559625 MBq in Davy 420 CR2 at any one time, which is lower than 1/10th total ALI (0.63 MBq - 630000 Bq) and below the maximum recommended bench top activity (2 MBq). Following the exposure, glassware (i.e. beakers) will be rinsed 3 times with DI water, this will be siphoned off into the sealable plastic container (carboy) and taken to Room 110 for disposal. Beakers will then be taken down to room 110 for a further rinse; they will then be acid washed in room 422. All working areas will be monitored for contamination before and after all radioactive exposures, suitable records will be kept.

5 Sampling

At the end of the exposure period, mussels (109 individual's total) will be dissected into specific tissue (digestive gland and gills), shell and all other soft tissue will be discarded. Any waste mussel samples will be placed in a sealed

plastic carboy in Davy 314C, this will then be stored for 3/4 months before disposal as non-hazardous lab waste in yellow bags, in accordance with the radioactive substance act 1993. All waste will be checked using a suitable detector prior to disposal. This will remain well below the university storage limits of 1 GBq.

5.1 Determination of genetic and molecular alterations

Following tissue extraction, a subsection of gill and digestive gland will be stored in RNAlater for future molecular work. Samples will be stored in the isotope store fridge until use. The remaining gill and digestive gland will be utilised for the comet, γ -H2AX, trypan blue (cell viability) and MN assays. All genetic bioassays will be performed in CR2, 314c or the CORIF lab. All equipment required (centrifuge, incubator) will be moved into appropriate room prior to use.

6 Transportation of samples to the University of Exeter

Samples, in individual scintillation vials will be packed in scintillation boxes and placed in sealed plastic bags, labelled with trefoil warning labels, surrounded by blue roll. A layer of vermiculite will line the bottom of the box as a precaution. This will then be placed into sealed, plastic boxes with warning labels on the inside. Water samples (50 total) will have a maximum radiation activity of 999 Bq. This is calculated from the following: 12 (1 mL in duplicate – so two per beaker) water samples taken on days 1 and 9 – totalling 999 Bq (not accounting for decay. In accordance with the Regulations for the safe transport of radioactive material, SSR-6 (2012 edition), which state that the activity limit for an exempt consignment (^{32}P specific) is 100000 Bq, samples will be transported to the University of Exeter

by the researcher via car. Samples will be brought back to Plymouth and disposed of as shown below. In regards to transportation, the driver will;

1. be aware of contents and will know what actions to be taken in the unlikely event of a spill,
2. ensure contact details and copy of the Local Rules are available with the package for the emergency services should they not be able to talk with the driver,
3. ensure relevant source records are updated,
4. monitor packages for both surface dose rate and contamination before dispatch and recording the values,
5. produce a document to accompany the package that details the contents,
6. ensure that there is sufficient data that proves the activity levels are within the exempt limits stated,
7. ensure that the receiving facility has the necessary Permits and facilities to keep and work with the material and
8. ensure the RPS is aware of the proposals and comments as necessary.

7 Disposals

The entire experimental procedure for the researcher to be undertake under these LRs is noted above. The university's monthly aqueous disposal limit for ^{32}P is 50 MBq, maximum theoretical disposal for the entire experimental procedure covered by these Local Rules (assuming one batch of standard solution as defined in section 3 is purchased per experiment and entire stock disposed of each time) would be 9.25 MBq, which is well under this limit. Small amounts of solid radioactive waste (waste tissue), sealed scintillation vials and other contaminated apparatus (i.e. pipette tips) will be stored in a plastic bag in the

314C freezer for around two months (4.5 half-lives) before disposal as solid waste in Davy 110. The levels of activity associated with the solid waste will be below the bi-monthly disposal limit of 40 KBq (The sum total of kBq of all other radionuclides in any one item of waste does not exceed 40). All disposals will be recorded and decay corrected to the certificate date to ensure records are accurate and comparable.

Disposals will be made of analysed solutions at the end of each experimental batch run. New experiments will not be started until all disposals from prior experiment have been undertaken and appropriate records made and approved by the RMS and CORIF laboratory manager (Dr Alex Taylor). Under no circumstances, will samples that have been analysed be stored in cupboards or bench space in the Davy 108, 110, 110A laboratory suite.

8 Restricting exposure

See RPD I

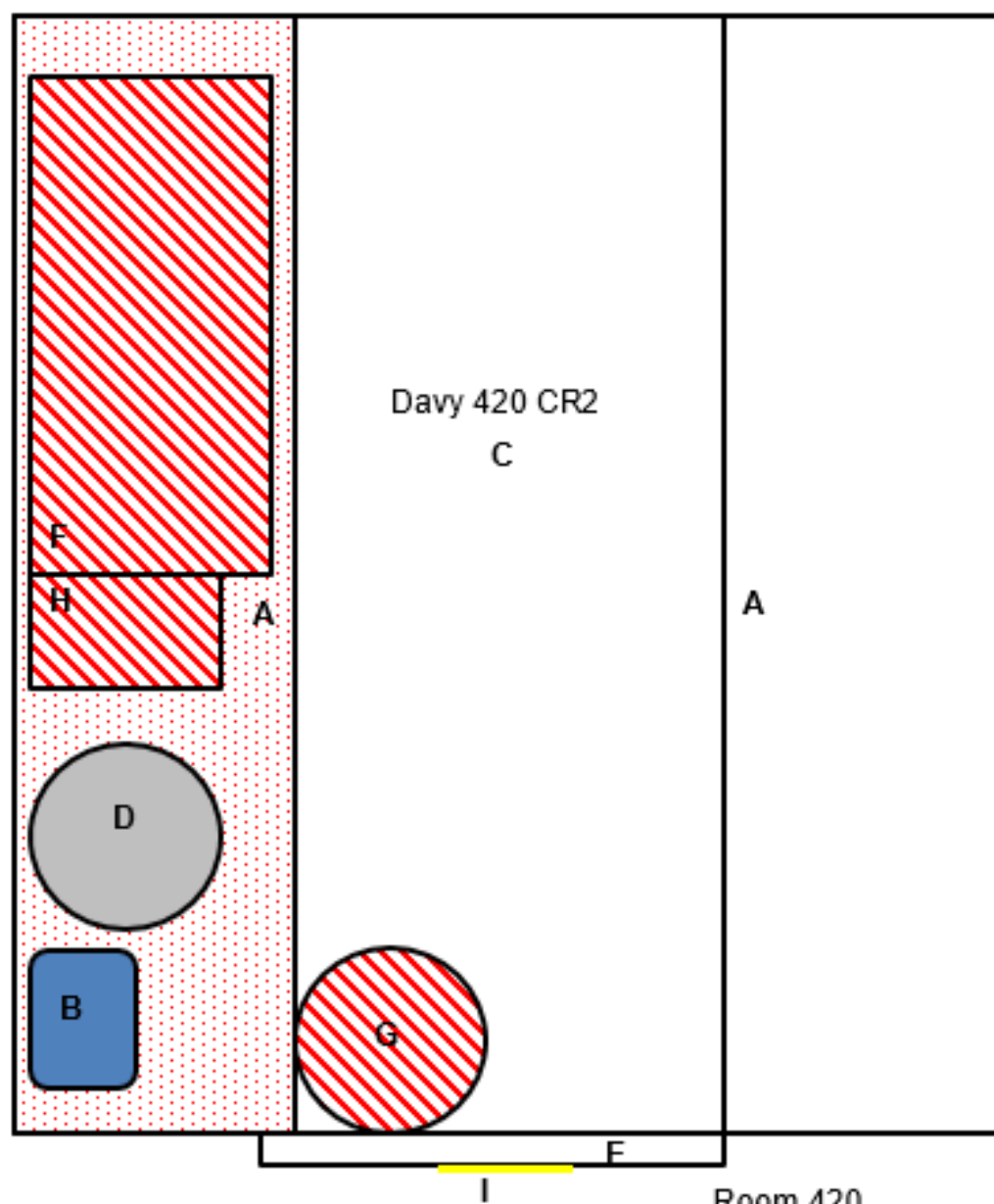
9 Contingency arrangements

See RPD I

List of accepted publications

1. Vernon, EL., Smith, JT., Jha, AN. 2018. Relative comparison of tissue specific bioaccumulation and radiation dose estimation in marine and freshwater bivalves following exposure to phosphorus-32. Journal of Environmental Radioactivity. 192. 312-320.10.1016/j.jenvrad.2018.07.005

DAVY 420 (CR2) RADIATION HAZARD MAP



KEY

A	Laboratory bench (left) or shelving unit (right)
B	Sink – No radioactive water to be poured down here
C	Walkway
D	Freshwater butt
E	Sliding door (locked/labelled) when running radioactive exposures)
F	P-32 acrylic shielding – all exposures carried out within shielding
G	Sealed plastic carboy – radioactive water to be stored in here until disposal
H	Storage of working stock solutions and waste (pipette tips etc.) in tray
I	Printed hazard map and safety instructions (appropriate COSHH forms)
	Radioactive materials sometimes present (during exposure)– door locked/labelled with trefoil when radioactive exposures are running
	Radioactive materials sometimes present – short duration during tissue dissection

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